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MICROPROPAGATION

Mesos components ($CaCl_2$, $MgSO_4$, KH_2PO_4) are critical for improving pear micropropagation

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Abstract Pear accessions and species show a broad response to tissue culture media due to the wide genetic diversity that exists in the available pear germplasm. An initial study of mineral nutrition using a systematic response surface approach with five Murashige and Skoog medium mineral stock solutions indicated that the mesos factor ($CaCl_2$, $MgSO_4$, and KH_2PO_4) affected most plant responses and genotypes, suggesting that additional studies were needed to further optimize these three mesos components for a wide range of genotypes. Short stature, leaf spots, edge necrosis, and red or yellow coloration were the main symptoms of poor nutrition in shoot cultures of 10 diverse pear genotypes from six species. A surface response experimental design was used to model the optimal factor and factor levels for responses that included overall quality, leaf character, shoot multiplication, and shoot height. The growth morphology, shoot length, and multiplication of these pear shoots could be manipulated by adjusting the mesos components. The highest quality for the majority of genotypes, including five *P. communis* cultivars, *P. koehnei*, *P. dimorphophylla*, and *P. pyrifolia* 'Sion Szu Mi', required higher concentrations (>1.2× to 2.5×) of all the components than are present in Murashige and Skoog medium. 'Capital' (*P. calleryana*) required high $CaCl_2$ and $MgSO_4$ with low KH_2PO_4; for 'Hang Pa Li' (*P. ussuriensis*), low $CaCl_2$ and moderate to low $MgSO_4$ and KH_2PO_4 produced high-quality shoots. Suitable combinations of the meso nutrients produced both optimum shoot number and shoot length in addition to general good plant quality.

Keywords Calcium · Magnesium · Media optimization · Phosphorous · *Pyrus*

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Introduction

Micropropagation has an important role in the rapid production and utilization of new horticultural crop cultivars. Micropropagation also provides a means to store germplasm and maintain disease-free stocks (Reed *et al*. 2011). For successful propagation and *in vitro* storage, choosing the correct growth medium is one of the most important steps in developing a useful protocol. Development of an appropriate culture medium for a specific crop can be quite complex because the response to the culture medium is often genotype-dependent and the effects of mineral nutrition on morphogenesis are poorly understood (Ramage and Williams 2002; Greenway *et al*. 2012). Many slow-growing or recalcitrant species and cultivars do not respond to the classical optimization approach of testing plant growth regulators (PGRs) or screening existing medium formulations such as Murashige and Skoog (MS; Murashige and Skoog 1962) or Woody Plant

medium (Lloyd and McCown 1980). Due to the wide genetic diversity found in pear germplasm collections, there are also diverse growth responses to various media (Bell and Reed 2002; Bell *et al*. 2009; Nakajima *et al*. 2012), and many pear species and cultivars are difficult to grow on any of the standard tissue culture media (Reed *et al*. 2012).

Minerals play an important role in the regulation of both plant morphogenesis and growth (Ramage and Williams 2002). Nutrient deficiencies are well studied in field plants (Bennett 1993), but are not as commonly studied *in vitro*. Preece (1995) noted that plant growth on suboptimal nutrient media may be compensated for by higher PGR concentrations, and media with optimal nutrients may require lower PGR concentrations for good plant growth.

Determining the mechanisms of nutrient availability that control plant growth is a major challenge in plant biology, and the interactions that affect the uptake of important nutrients make it even more challenging (Hermans *et al*. 2010). MS medium was developed for tobacco callus by testing single variables (Murashige and Skoog 1962). Multifactor design experiments provide much more information about factor effects than can be obtained by testing factors one at a time (Niedz and Evens 2007). Based on the analysis of five mineral nutrient factors in MS salts [NH_4NO_3, KNO_3, mesos ($CaCl_2$, $MgSO_4$, and KH_2PO_4—nutrients needed at intermediate concentrations), micros, and Fe] on *in vitro* growth of five pear types using a multifactor surface response design, the mesos nutrient component was the most influential (Reed *et al*. 2013). The mineral nutrients also affected morphogenesis. For example, relative to shoots grown on MS medium, shoots grown on media with high iron concentrations were always stunted, while those on high-mesos media grew vigorously and produced large leaves. Shoot multiplication could also be manipulated with mineral nutrients without changing the PGRs.

To improve the growth of *in vitro* pear shoots, the objective of this study was to determine the effects of the three mesos components ($CaCl_2$, $MgSO_4$, KH_2PO_4) on the growth of a diverse collection of pear germplasm at the National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon. A response surface approach was used to determine the optimum nutritional requirements of pears for these three salts.

Materials and Methods

Culture conditions. Ten pear genotypes from six species were chosen because they did not grow well on MS medium (Table 1) and represent a diverse range of pear germplasm. Shoot cultures were grown in Magenta GA-7 tissue culture boxes (Magenta Corp., Chicago, IL) with 40 ml medium per box of a modified MS medium selected from the earlier experiment, which contains (per liter) 4.4 μM N^6-benzyladenine (PhytoTechnology Labs, Shawnee Mission, KS), 3 g agar (Phytotech A111, PhytoTechnology Labs), and 1.75 g Gelrite (PhytoTechnology Labs) at pH 5.7. Cultures were grown at 25°C under a 16-h photoperiod with 70–90 μM m^{-2} s^{-1} irradiance provided by a combination of cool- and warm-white fluorescent bulbs and transferred to new medium every 3 wk.

Table 1. Identifying number, name, and taxon of 10 pear genotypes tested with the three mesos factors

Local ID	Genotypes	Species
2384.001	Ayers	*P. communis* L.
662.001	Capital	*P. calleryana* Decne.
268.001	Hang Pa Li	*P. ussuriensis* M.
2933.001	Horner 51	*P. communis* L.
367.001	Luscious	*P. communis* L.
1345.001	Old Home×Farmingdale 87 (OH×F87)	*P. communis* L.
1214.001	*P. dimorphophylla*	*P. dimorphophylla* Mak.
815.001	*P. koehnei*	*P. koehnei* C. K. Schneider
532.002	Sion Szu Mi	*P. pyrifolia* Burm.
1164.001	Winter Nelis	*P. communis* L.

Experimental approach. The study was designed to determine the effects and optimal concentrations of the meso nutrients $CaCl_2$, $MgSO_4$, and KH_2PO_4 in MS medium. A response surface experimental design was used (Myers and Montgomery 2002; Table 2). The D-optimal design was augmented to include seven additional points for detecting additional signal (*i.e.*, lack-of-fit analysis or curvature) possibly not captured in the design. Six points were replicated to estimate the pure error through the design space; this included one point replicated twice for MS medium as it was a coordinate within the three-dimensional design space. The replicates provided a statistically valid estimation of the pure error of this experiment. For each genotype, an experimental "run" included 10 shoots, each with two nodes (approximately 10 mm), the apical section removed, with five shoots planted in duplicate magenta boxes (n=10). Five replicated points (runs 2 and 11, 3 and 10, 4 and 8, 12 and 21, and 14 and 18) were included as a second set of duplicate magenta boxes each containing five shoots (Table 2). The remaining salts were at MS medium concentrations. Boxes were randomized on the growth room shelf. Each group of shoots was transferred to a fresh box of the same medium at 3-wk intervals. Shoots were harvested after three passages (9 wk) of culture on the same medium.

Data. Seven responses were measured at each of the design points: (1) a subjective rating of plant appearance (1=poor quality, 2=acceptable quality, 3=good quality; Niedz and Evens 2007); (2) shoot length (longest shoot measured in

Table 2. Treatment combinations and control treatments 22 and 23 used for the three-factor mesos study

Run	Factor 1 $CaCl_2$ (×MS)[z]	Factor 2 $MgSO_4$ (×MS)[z]	Factor 3 KH_2PO_4 (×MS)[z]
1	1.5	1.5	2.5
2	0.5	0.5	2.5
3	0.5	2.5	0.5
4	2.5	0.5	0.5
5	2.5	2.5	2.5
6	1.5	2.5	0.5
7	2.5	2.5	1.5
8	2.5	0.5	0.5
9	0.5	1.5	1.5
10	0.5	2.5	0.5
11	0.5	0.5	2.5
12	0.5	2.5	2.5
13	2.5	1.5	0.5
14	2.5	0.5	2.5
15	2.5	2.5	0.5
16	1.5	1.5	1.0
17	0.5	0.5	0.5
18	2.5	0.5	2.5
19	1.0	2.0	1.5
20	1.5	0.5	1.5
21	0.5	2.5	2.5
22	1.0	1.0	1.0
23	1.0	1.0	1.0

[z] Level of factor relative to that found in MS medium

millimeters); (3) shoot multiplication (number of shoots); (4) leaf color (1=green, 2=pale green, 3=pink-edged, 4=red or brown); (5) leaf spotting/necrosis (1=absent, 2=minor, 3=major); (6) callus (1=absent, 2=callus ≤3 mm, 3=callus >3 mm); and (7) leaf size (1=small, 2=medium, 3=large). Three shoots were sampled from each box in a predetermined pattern (two corners and the center on a diagonal from the label) to avoid subjective selection (n=6). The remainder of the shoots were photographed (n=4). Visible physiological disorders such as hyperhydricity and abnormal growth forms (hyperplasia, fasciation, epinasty, etc.) were noted. Design-Expert software optimization criteria were set as follows: quality and shoot length=maximum, shoot number=3, leaf size=2, and leaf spot and leaf color=minimum.

Statistical analysis. Experimental design and point selection, polynomial equation generation, analysis of variance (ANOVA), and graphical displays were calculated using Design-Expert 8 software (Design-Expert 2010). The experimental design was a three-factor response surface design with blocking. Genotype was not included as a factor. The concentrations of $CaCl_2$, $MgSO_4$, and KH_2PO_4 ranged from 0.5× to 2.5× MS levels. Design points were selected using modified D-optimal criteria suitable for fitting a quadratic polynomial (Niedz and Evens 2006, 2007; Evens and Niedz 2008).

Results

All three mesos components produced significant effects for the responses evaluated. The models were highly significant for many responses for most genotypes (Table 3). Representative ANOVA analyses (Electronic Supplementary Material (ESM) 1) and complete graphical data and photographs (ESM 2) are available as online appendices. The graphical data are modeled based on shoot cultures grown at each design point, and actual design point data are shown as red dots on some graphs.

Quality rating. For quality rating, the model was significant for genotypes "Capital," "OH×F87," 'Sion Szu Mi,' and "Winter Nelis" (Table 3). Seven of the 10 genotypes responded to at least one meso component. Better quality was produced with high $CaCl_2$ and $MgSO_4$ and with low KH_2PO_4 for "Capital" (*P. calleryana*; Fig. 1*A*, *E*) or all three factors at moderate concentrations for 'Hang Pa Li' (*P. ussuriensis*; Fig. 1*B*, *G*). The majority of genotypes, including the *P. communis* cultivars, *P. koehnei*, *P. dimorphophylla*, and *P. pyrifolia* 'Sion Szu Mi' required substantially higher concentrations of $CaCl_2$ and KH_2PO_4 than the MS medium and moderate to high $MgSO_4$, although in some cases $MgSO_4$ was not significant for improved quality, as illustrated by 'OH×F87' (Fig. 1*C*, *I*). Plant morphology varied greatly with the species depending on the combination of components, resulting in poor or good growth (Fig. 1). Low levels of all the mesos produced poor growth for 'Capital' (Fig. 1*D*), while low $CaCl_2$ resulted in stunted growth and discolored leaves for 'OH × F87' and 'Hang Pa Li' (Fig. 1*F*, *H* and ESM 2).

Shoot number. The effect of mesos components on shoot multiplication varied widely. Only *P. koehnei* and 'Winter Nelis' had significant models for increased shoot number, but seven genotypes had at least one factor that was significant (Table 3). 'Capital' produced the most shoots with low KH_2PO_4 and high $CaCl_2$ regardless of $MgSO_4$ (Fig. 2*A*). *P. koehnei* usually produced excessive tiny shoots that did not elongate, but more moderate shoot numbers were seen with minimal KH_2PO_4 and $MgSO_4$ combined with moderate $CaCl_2$. 'Horner 51', which usually remained as a single shoot and did not often produce multiple shoots, required low levels of all mesos for the most shoot production

Table 3. ANOVA summary of six responses to three mesos components ($P \leq 0.05$) for the 10 pear genotypes

Genotype	Factor	Quality	Shoot number	Shoot length	Leaf size	Leaf spots	Leaf color
Ayers	Model	NS	NS	0.0321	NS	0.0464	0.0099
	$CaCl_2$	NS	0.0170	0.0008	NS	NS	NS
	$MgSO_4$	NS	NS	NS	NS	NS	0.0139
	KH_2PO_4	NS	NS	NS	0.0257	NS	0.0021
Capital	Model	0.0051	NS	0.0003	0.0318	NS	0.0215
	$CaCl_2$	NS	NS	0.0001	0.0058	NS	NS
	$MgSO_4$	0.0019	NS	NS	0.0078	0.0056	0.0026
	KH_2PO_4	0.0026	0.0225	0.0033	NS	NS	0.0417
Hang Pa Li	Model	NS	NS	NS	NS	NS	0.0352
	$CaCl_2$	NS	0.0225	NS	NS	NS	NS
	$MgSO_4$	NS	NS	NS	NS	NS	0.0048
	KH_2PO_4	NS	0.0167	NS	NS	NS	NS
Horner 51	Model	NS	NS	NS	0.0016	0.0363	NS
	$CaCl_2$	NS	0.0419	NS	NS	NS	NS
	$MgSO_4$	NS	NS	NS	0.0464	0.0357	0.0082
	KH_2PO_4	0.0334	0.0319	NS	<0.0001	0.0055	0.0201
Luscious	Model	NS	NS	0.0451	NS	0.0204	0.0146
	$CaCl_2$	0.0023	NS	0.0035	NS	0.0078	NS
	$MgSO_4$	NS	NS	NS	0.0302	0.0036	0.0169
	KH_2PO_4	NS	NS	NS	NS	0.0078	0.0003
OH×F87	Model	0.0026	NS	0.0173	0.0003	0.0005	NS
	$CaCl_2$	0.0005	NS	0.0004	<0.0001	0.0057	NS
	$MgSO_4$	NS	0.0033	NS	0.0003	0.0044	NS
	KH_2PO_4	0.0113	NS	NS	0.0037	0.0002	0.0055
P. dimorphophylla	Model	NS	NS	NS	NS	NS	NS
	$CaCl_2$	NS	NS	NS	NS	NS	NS
	$MgSO_4$	NS	NS	NS	0.0275	NS	NS
	KH_2PO_4	NS	NS	NS	NS	NS	NS
P. koehnei	Model	NS	0.0002	NS	NS	NS	NS
	$CaCl_2$	NS	NS	0.0500	NS	NS	NS
	$MgSO_4$	NS	NS	NS	0.0411	NS	NS
	KH_2PO_4	0.0365	<0.0001	NS	NS	NS	NS
Sion Szu Mi	Model	0.0474	NS	NS	NS	NS	0.0212
	$CaCl_2$	NS	NS	NS	NS	NS	NS
	$MgSO_4$	0.0130	NS	NS	NS	NS	0.0018
	KH_2PO_4	NS	NS	NS	NS	0.0154	0.0072
Winter Nelis	Model	0.0098	0.0238	NS	0.0308	0.0058	0.0025
	$CaCl_2$	0.0024	NS	0.0053	NS	NS	NS
	$MgSO_4$	0.0251	0.0045	NS	0.0032	NS	0.0385
	KH_2PO_4	0.0020	NS	NS	NS	<0.0001	0.0002

(Fig. 2*B*). 'OH×F87' and 'Winter Nelis' required low $MgSO_4$ and high KH_2PO_4, while the $CaCl_2$ concentration was not a significant factor for this trait (Fig. 2*C*).

Shoot length. The model was significant for four genotypes and $CaCl_2$ was significant for six genotypes (Table 3). "Capital" had a rosette-type growth with multiple shoots that did not elongate; the tallest (~22 mm) was seen with moderate $MgSO_4$, moderate to low KH_2PO_4, and high $CaCl_2$ (Fig. 2*D*). 'Luscious' and 'Winter Nelis' required high concentrations of all three factors for the longest shoots (Fig. 2*F*). For 'Horner 51', *P. dimorphophylla*, and 'Sion Szu Mi', none of the factors were significant (Fig. 2*E*).

Leaf size. The leaf size model was significant for four genotypes, while eight had at least one significant factor

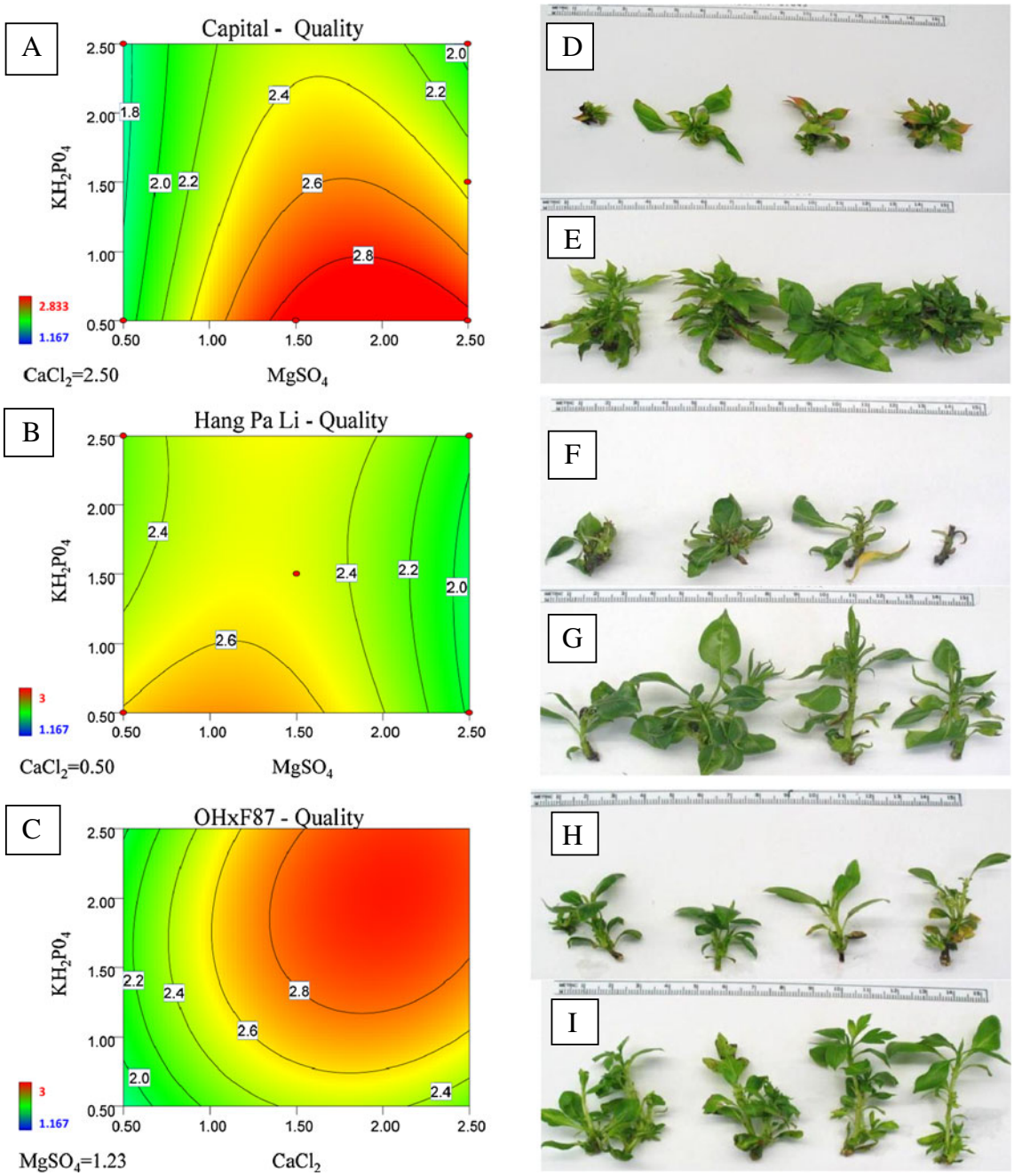

Figure 1. *A–C*; Responses of representative pear genotypes to mesos treatments. Subjective shoot quality rating (1: low to 3: high) on each treatment used by Design-Expert software to project the "best quality" shoots: *P. calleryana* 'Capital' (treatment 20 (*D*); treatment 15 (*E*)); *P. ussuriensis* 'Hang Pa Li' (treatment 12 (*F*); treatment 16 (*G*)); *P. communis* "OH×F87" (treatment 12 (*H*); treatment 20 (*I*)). Treatments are as shown in Table 1. *Red dots* indicate actual design points.

(Table 3). Generally, when all three factors were greater than 1.5× MS, the shoots produced large leaves (data not shown). The most desirable leaf size for tissue culture plants is not always the largest, however, so optimal leaf size for analysis was targeted as "medium" (rating of 2). 'Horner 51' had moderate leaf size with moderate $CaCl_2$, low $MgSO_4$, and high KH_2PO_4 (Fig. 3*A*). For 'Luscious', moderate $CaCl_2$ and KH_2PO_4 with low $MgSO_4$ produced medium-sized leaves (Fig. 3*B*); for 'OH×F87', low $CaCl_2$, moderate $MgSO_4$, and KH_2PO_4 was best (Fig. 3*C*).

Leaf spot and necrosis. The model was significant for five genotypes and at least one factor for six genotypes (Table 3). The *P. communis* cultivars 'OH×F87' and 'Luscious' had substantially improved leaf quality as all three mesos factors increased (Fig. 3*D*). *P. dimorphophylla* had the opposite

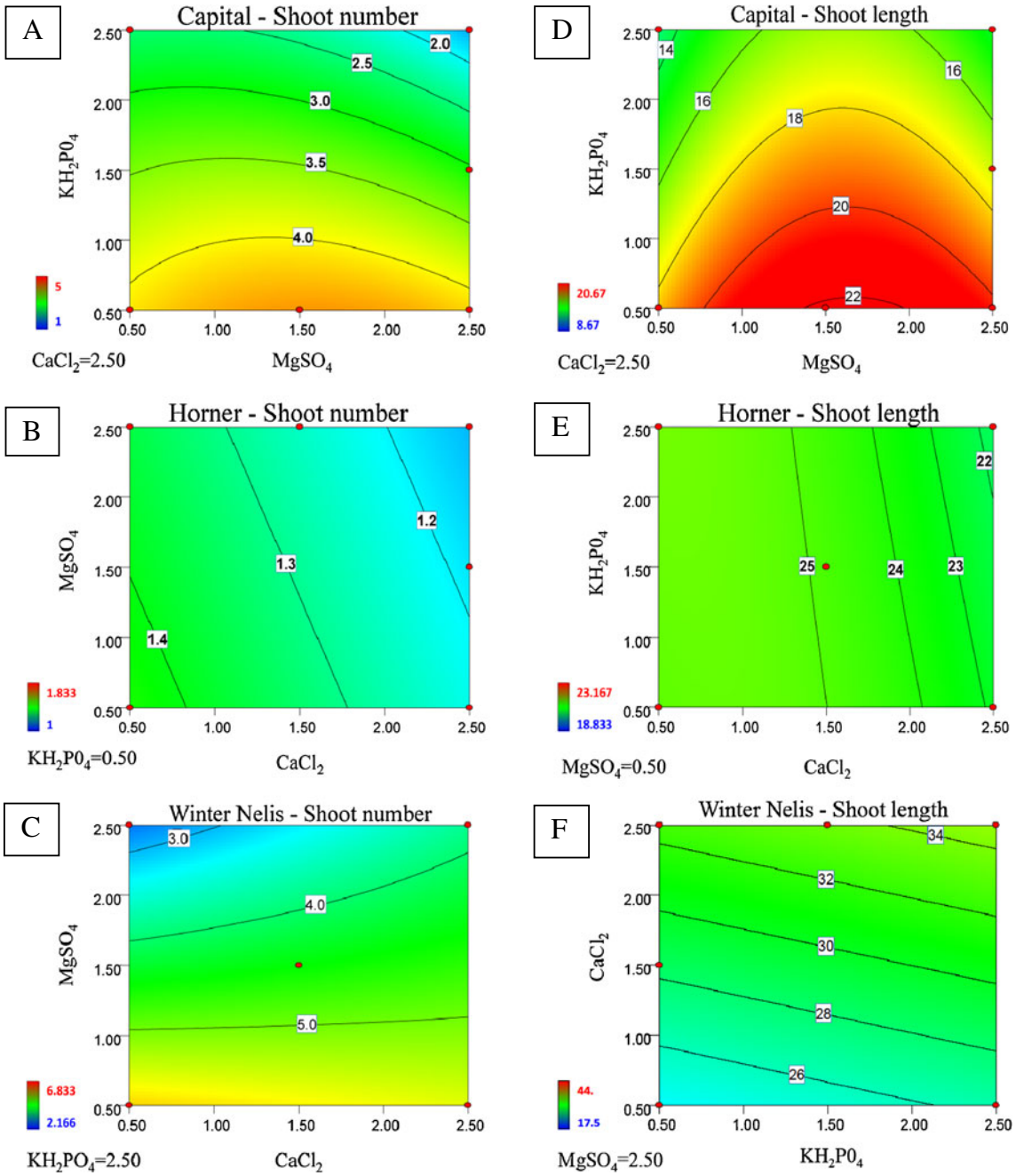

Figure 2. Responses of representative pear genotypes to mesos treatments. Shoot number (*left*) (*A–C*) and shoot length (*right*) (*D–F*) of pears showing three patterns of response. *Red dots* indicate actual design points.

response; at the lowest concentration of all three factors, leaf spot symptoms were reduced (Fig. 3*E*). *P. koehnei* showed the fewest spots with the highest $MgSO_4$ and the lowest KH_2PO_4 regardless of $CaCl_2$ concentration (Fig. 3*F*).

Leaf color. The model was significant for six genotypes; eight had at least one factor that was significant (Table 3). $CaCl_2$ concentration was not significant for leaf color for any of the 10 genotypes. Most genotypes showed better leaf color as $MgSO_4$ and KH_2PO_4 increased, although exceptions were seen with *P. koehnei* (low KH_2PO_4) and 'Winter Nelis' (low $MgSO_4$; ESM 2).

Projected optimums. Optimal mesos concentrations were projected for each genotype by the following two techniques: treatments selected using a subjective quality rating of cultured shoots and projections based on Design-Expert software using defined criteria for the "ideal plant" (Table 4). The projections from Design-Expert software employed several optimum factors, and those optimums,

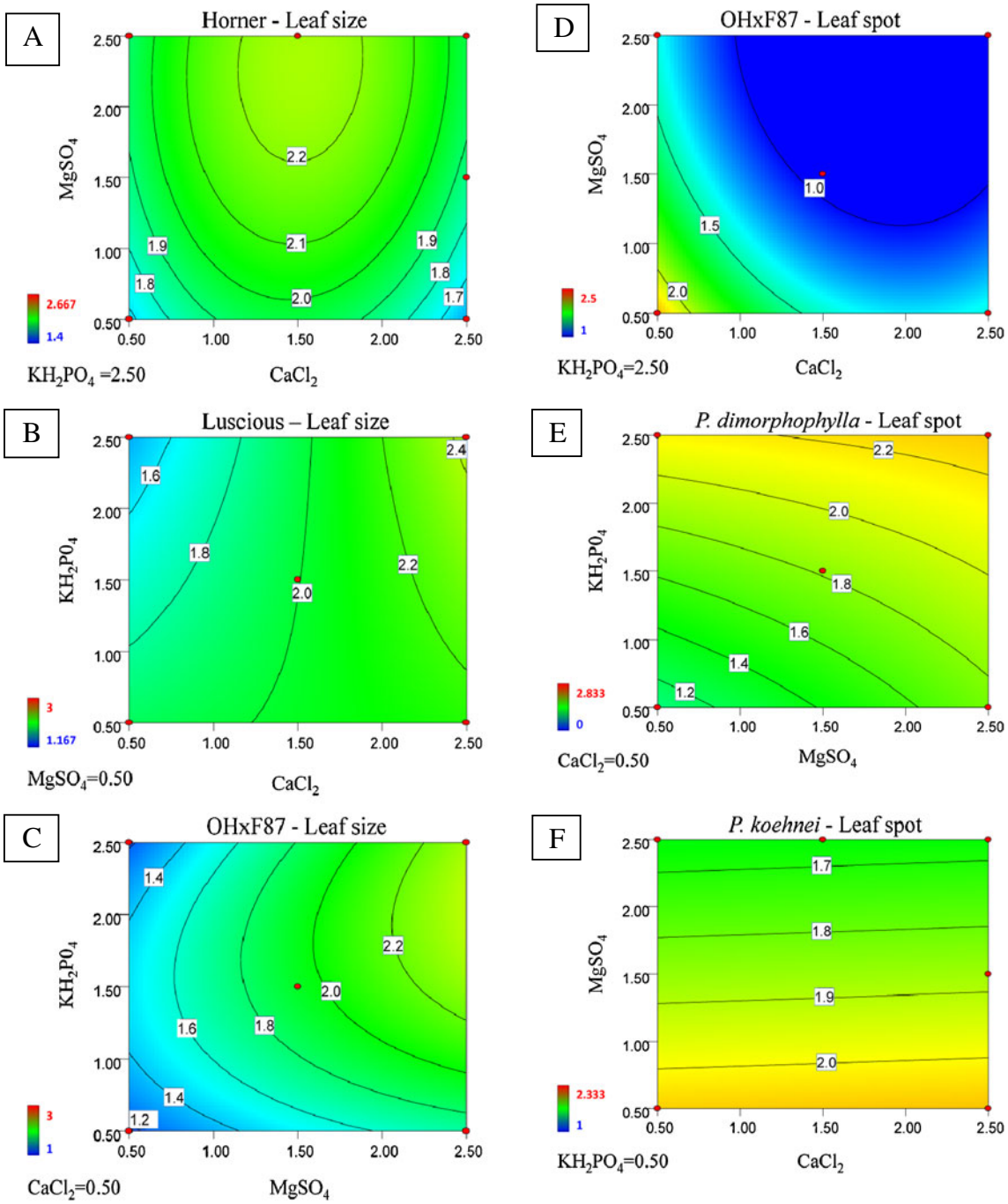

Figure 3. Responses of representative pear genotypes to mesos treatments based on ratings from 1 (small or low) to 3 (large or high). *Left column* (*A–C*), leaf size (2=medium size). *Right column* (*D–F*), leaf spot/necrosis (1=no leaf spots). *Red dots* indicate actual design points.

when compared to the region of the best quality from the subjective quality rating graphs, were usually similar. The projected optimums were not tested on shoot cultures. In most cases, two or three of the mesos factors were involved in improving the response of each genotype (Table 3). Increasing all of the mesos factors eliminated leaf spots and necrosis for many *P. communis* genotypes including 'Ayers', 'Luscious', 'OH×F87', and 'Winter Nelis', while moderate improvements in overall quality and reduction in physiological symptoms were seen with *P. dimorphophylla* and *P. koehnei* (ESM 2).

Physiological disorders. Physiological disorders such as epinasty, shoot tip necrosis, hypertrophy, fasciation, hyperhydricity, and callus production were observed over a range of treatments (data not shown). Treatments with low

Table 4. Projected best concentration of each meso component for each genotype from the actual quality ratings compared to the optimizations projected by Design-Expert based on criteria for the "ideal plant"

Species	Genotype	Concentration based on quality rating			Optimization by Design-Expert[z]		
		$CaCl_2$	$MgSO_4$	KH_2PO_4	$CaCl_2$	$MgSO_4$	KH_2PO_4
P. calleryana	Capital	2.5	≥1.5	≤1.0	2.2	1.9	0.5
P. communis	Ayers	>2.0	1.5	2.5	2.0	1.2	1.8
P. communis	Horner 51	2.5	2.5	2.5	2.2	2.5	0.8
P. communis	Luscious	2.5	>2.0	>2.0	2.0	2.0	2.0
P. communis	OHxF87	≥1.5	1.2	≥1.5	2.5	1.0	2.5
P. communis	Winter Nelis	>2.0	≥1.5	2.5	2.1	1.5	2.1
P. dimorphophylla	*P. dimorphophylla*	≥1.5	>2.0	2.5	2.5	2.5	2.5
P. koehnei	*P. koehnei*	>2.0	2.0	2.5	1.4	1.8	2.1
P. pyrifolia	Sion Szu Mi	2.5	1.5	≥1.5	2.0	2.0	2.0
P. ussuriensis	Hang Pa Li	0.5	≤1.5	≤1.0	0.5	1.8	1.6

[z] Design-Expert predictions were not tested

concentrations of one or more factors, including the MS medium control, were more likely to produce disorders, but there was no general trend (data not shown). Epinasty was seen on low calcium with 'Ayers', 'Winter Nelis', and 'Hang Pa Li' (ESM 2B, F, J). Shoot tip necrosis was more evident with low calcium for 'Hang Pa Li', 'Luscious', and *P. dimorphophylla*. Hypertrophy was seen on a few treatments and fasciation was only seen on occasional shoots. Hyperhydricity was also evident when low levels of all three components were used in the medium; 'Luscious' and 'Winter Nelis' shoots are good examples (ESM 2D, F). Callus production was not significant for any factors or for any of the genotypes, but large amounts of callus were present on 'Ayers' and 'Winter Nelis' with low KH_2PO_4 and $MgSO_4$ combined with medium to high $CaCl_2$, on 'Sion Szu Mi' with high KH_2PO_4 and low $MgSO_4$ and $CaCl_2$, and on *P. koehnei* with medium KH_2PO_4 and $MgSO_4$ and the MS level (1×) of $CaCl_2$ (data not shown). The remaining genotypes had little or no callus for any treatments.

Discussion

The wide use of MS medium as a standard growth medium for all types of plant tissue culture has given the false impression that many plants are difficult to culture if they will not grow well on MS medium. MS medium was developed for the rapid growth of tobacco callus cultures (Murashige and Skoog 1962) and not for the organogenesis needed for micropropagation (Adelberg *et al*. 2010). Our experience with diverse pear genotypes confirms that the so-called standard media require nutrient optimization for the micropropagation of unique groups of plants. Our initial studies found that the mesos component of the MS medium was inadequate for good growth and multiplication of several pear species (Reed *et al*. 2013). This current study modeled the effect of each of the three components on pear micropropagation and expanded the range of species and cultivars tested. Using a response surface experimental design approach allowed us to model each cultivar's response throughout the three-dimensional design space defined by the three meso salts and their concentration ranges by using only 23 points (Table 2); this is one advantage of this type of approach.

Analysis of spent media of *Hemerocallis* shoot cultures led Adelberg *et al*. (2010) to conclude that reformulation of MS medium might improve the availability of Ca, P, and Mg by increasing solubility. Our finding agrees with their assumption that MS medium does not enhance organogenesis or morphogenesis. Successful optimization of the mineral components of the culture medium may also allow a reduction in the concentrations of PGRs required in the growth media (Preece 1995). In this current study, most of the genotypes had better overall quality (Fig. 1*E*, *G*, *I* and ESM 2), longer shoots (Fig. 2 and ESM 2), and better leaf quality (Fig. 3 and ESM 2) with increased concentrations of some or all three mesos chemicals. This improved growth came without changes to the PGR concentrations.

Calcium is a major mediator of physiological processes in plant cells and may be a limiting factor in plant tissue culture (Williams 1993). This was shown in suspension cultures of carrot (*Daucus carota* L.; Jansen *et al*. 1990) and *Eucalyptus urophylla* (Arrudal *et al*. 2000) where increasing Ca concentrations in the medium doubled the number of somatic embryos produced. Ca appeared deficient in *Hemerocallis* shoot cultures grown on MS medium, and the actual uptake of Ca varied with the genotype (Adelberg *et al*. 2010). Ca is highly important in cell division, cell wall formation, and meristem growth (Ramage and Williams

2002). Ca was the key component of several media developed for bromeliads and had a significant effect on the utilization of several other nutrients (Aranda-Peres *et al*. 2009). For *Aechmea nudicaulis* bromeliads, leaf mineral analysis indicated that the plantlets grown on 0.5× MS (1.5 mM Ca) were deficient in Ca, Mg, and Cu. After modifying the growth medium, the N, K, Zn, Mg, and B concentrations increased in leaves as Ca increased to 12 mM (4× MS). In our study of pear, high concentrations of $CaCl_2$ were required for good shoot elongation for 6 of 10 genotypes (Table 3). Increased $CaCl_2$ contributed to improved plant quality for 9 of the 10 pear genotypes in this study (Table 4) and was a significant factor for shoot length, shoot number, and some leaf responses (Table 3). The calcium deficiency symptoms of scorched leaf margins, epinasty, and shoot tip necrosis were corrected by increased calcium in the best treatments for many genotypes (Fig. 1 and ESM 2).

Magnesium is required for plant growth and is at the center of the chlorophyll molecule (Hermans *et al*. 2004, 2010). Mg deficiency symptoms include a yellowing of the leaf between the leaf veins, first appearing in older leaves and progressing to the younger leaves (Bennett 1993; Hermans *et al*. 2010). These leaf symptoms are very common in micropropagated pears, and we routinely observed them in many genotypes. All of the pears in this study had improved quality with ≥1.5× MS $MgSO_4$ concentrations (Table 4). Leaf color was significantly affected by $MgSO_4$ for 7 of the 10 genotypes, and leaf spot/necrosis symptoms were significantly reduced or eliminated on 4 of the 10 genotypes (Table 3). Magnesium concentrations in MS medium were also found to be too low for *Hemerocallis* shoot cultures, especially those grown on high sucrose and at high density (Adelberg *et al*. 2010).

Phosphorus is often a limiting factor in tissue culture media, and the MS medium formulation may not adequately supply rapidly growing shoots (Williams 1993; Adelberg *et al*. 2010). High-density cultures were P-deficient, and P availability also affected the transfer of high-density *in vitro*-grown plants to acclimatization in mist beds or greenhouses (Adelberg *et al*. 2010). In tobacco cultures, over half of the P was utilized after 20 d during shoot meristem initiation, while the remaining half was utilized for shoot production after 15 additional days of growth (Ramage and Williams 2002). Tobacco tissue culture media with high levels of phosphate produced fewer shoots, and the most shoots were produced on the standard MS concentration (1.25 mM; Ramage and Williams 2002).

Potassium is also required for plant growth and metabolic processes. There is a cytoplasmic requirement for K for protein synthesis and also to maintain turgor pressure (Leigh and Wyn Jones 1984). Higher K concentrations are required by fast-growing radishes compared to relatively slower-growing barley and ryegrass (Woodhouse *et al*. 1978). K deficiency substantially reduced plant growth during shoot development for *Hemerocallis* shoot cultures, but K concentrations in MS medium were generally suitable for growth (Adelberg *et al*. 2010). K is contained in our nitrogen source as well as in the mesos component, making analysis difficult. The effect of KH_2PO_4 on morphogenesis likely varies with plant type as we found that 4 of 10 genotypes produced the greatest shoot numbers on low KH_2PO_4, while the others were not significantly affected (Table 3 and ESM 2). KH_2PO_4 was not significant for shoot length, except for the rosette growth form of 'Capital' (*P. calleryana*) that produced increased shoot length with low KH_2PO_4.

The influence of mineral nutrition on culture morphogenesis is well documented (Preece 1995; Ramage and Williams 2002; Kintzios *et al*. 2004; Niedz and Evens 2007). Increasing all three of the mesos components in pear micropropagation medium significantly improved the growth of 8 of the 10 genotypes (four species; Table 3). Two species differed in their response to mesos for the best quality ratings; 'Capital' (*P. calleryana*) responded best to low KH_2PO_4 and 'Hang Pa Li' (*P. ussuriensis*) to low $CaCl_2$ and KH_2PO_4 and low to moderate $MgSO_4$. Pear shoots cultured on MS medium frequently produce callus tissue, leaf symptoms, and severe hyperhydricity (Bell *et al*. 2009). These symptoms were no longer problematic in shoots cultured on high-mesos medium, and plants showed improved overall quality (Fig. 1 and ESM 2).

The growth morphology, shoot length, and multiplication of pear shoots could be manipulated by adjusting the mesos components. Suitable combinations of the meso nutrients were determined that produced both an optimum shoot number and a reasonable shoot length in addition to general good plant quality (Table 4). When projecting an improved growth medium based on criteria set for the "ideal plant" using the Design-Expert software program (Design-Expert 2010), those analyses strongly agree with the quality data alone (Table 4). The subjective rating system for quality includes the responses taken as quantitative data and validates the use of rating systems in projecting the quality of micropropagated plants (Niedz *et al*. 2007).

The models produced in this study clearly indicated that all three of the mesos components of MS medium require adjustment for the best growth of a wide range of pear genotypes. The highest quality for the majority of genotypes, including the five *P. communis* cultivars, *P. koehnei*, *P. dimorphophylla*, and *P. pyrifolia* 'Sion Szu Mi,' required higher concentrations (>1.2× to 2.5×) of all the components than are present in MS medium. 'Capital' (*P. calleryana*) required high $CaCl_2$ and $MgSO_4$ with low KH_2PO_4; for 'Hang Pa Li' (*P. ussuriensis*), low $CaCl_2$ and moderate to low $MgSO_4$ and KH_2PO_4 produced high-quality shoots (Table 4). Mesos concentrations in pear culture media

significantly influenced overall shoot quality and height, leaf color, and physiological disorders. These factors can be adjusted as needed to produce the desired plant response for diverse pear species. The findings from this study are applicable to reducing physiological disorders and enhancing quality growth for other micropropagation systems.

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