Acta Physiol Plant (2015) 37:63
DOI 10.1007/s11738-014-1754-y

ORIGINAL PAPER

Screening genetically diverse pear species for in vitro $CaCl_2$, $MgSO_4$ and KH_2PO_4 requirements

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Received: 5 August 2014 / Revised: 21 October 2014 / Accepted: 16 December 2014 / Published online: 24 February 2015
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Abstract Conservation of important plant germplasm is often difficult due to the specific growth requirements of genetically diverse species including in vitro culture collections. Recently the mesos components ($CaCl_2$, $MgSO_4$, KH_2PO_4) of Murashige and Skoog medium were identified as one of the most influential groups of nutrients for five pear genotypes. To determine if this requirement also applied to a larger germplasm collection, 18 genotypes in six species were screened. Shoot quality, shoot length, leaf spots and leaf color were the most affected responses. Seven of nine *Pyrus communis* cultivars had improved shoot quality, five had significantly longer shoots, better leaf color and fewer leaf spots while two had more shoots. Two of the four *Pyrus pyrifolia* cultivars had improved shoot quality while three had better leaf color and fewer leaf spots. *Pyrus calleryana* 'Capital', *Pyrus cordata* and *Pyrus ussuriensis* 'Harbin' had longer shoots while *Pyrus koehnei* had less callus. *P. ussuriensis* 'Hang Pa Li' was the only genotype where shoot quality declined at high mesos concentrations. Quantitative ion analysis detected substantially higher concentrations of Ca, Mg and K, but significantly less Fe, in the shoots cultured on increased mesos compared to controls. This study confirms that increased mesos improved growth of *P. communis* and *P. pyrifolia* cultivars, but produced fewer significantly improved responses for four other species.

Keywords Growth medium · Mesos components · Micropropagation · Mineral nutrition · *Pyrus*

Abbreviations

Mesos	Components $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KH_2PO_4 (monobasic)
MS	Murashige and Skoog Medium

Introduction

International germplasm collections hold the genetic diversity of crop species for use in breeding. Maintaining backup collections in vitro or as cryopreserved shoot tips require healthy shoot cultures (Reed 1999). Shoot cultures can be held at cold temperatures (−1 to 4 °C) for one to three years or as shoot tips in liquid nitrogen for long-term

Communicated by P. K. Nagar.

Electronic supplementary material The online version of this article (doi:10.1007/s11738-014-1754-y) contains supplementary material, which is available to authorized users.

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storage (Reed et al. 1998, 2013a). Optimizing the growth of widely diverse genotypes requires attention to the nutritional differences among the species and cultivars. The mineral composition of growth medium critically affects plant morphogenesis including shoot growth and multiplication, abnormal growth and physiological disorders (Niedz and Evens 2007; Reed et al. 2013b, c). Plant species and cultivars are often specific in their mineral requirements, therefore no universal medium for in vitro culture is available (Skirvin 1981). A number of studies report the importance of mineral elements, suggesting a serious need to review nutrient medium composition (Bondarev et al. 2003; Bosela and Michler 2008; Bucher et al. 2007; Preece 1995; Sallanon et al. 1997).

It is well documented that it is difficult to grow many pear cultivars and species (Bell and Reed 2002). Pear medium formulations were developed to suit general growth for specific pear cultivars (Singha 1986; Zhao and Gu 1990). MS basal medium (Murashige and Skoog 1962) is the most widely used formulation at full or half strength, or with slight modifications. In addition to LP (Quoirin and Lepoivre 1977), DKW (Driver and Kuniyuki 1984), and WPM (Lloyd and McCown 1980) are also often employed (Bell et al. 2009). Regardless of such modifications and developments, the optimal pear culture medium for diverse genotypes is still not available (Reed et al. 2013b; Wada et al. 2013).

Our laboratory began to utilize a systematic modeling approach for pear medium development and identified the mesos components of MS ($CaCl_2$, $MgSO_4$, KH_2PO_4) and the nitrogen components as the most important driving factors for optimal in vitro pear growth (Niedz and Evens 2007; Reed et al. 2013b). A previous study found that most of the nine pear genotypes tested required higher concentrations of mesos (1.5–2.5×) for the best growth and shoot quality (Wada et al. 2013). Increased mesos in pear culture media significantly improved shoot quality, shoot height, leaf color with few physiological disorders. However, the applicability of increased mesos to the full range of pear germplasm was unknown.

The goal of this study was to screen a wider range of pear germplasm from the National Clonal Germplasm Repository (NCGR) for response to increasing mesos concentrations and the effect on tissue fresh weight and nutrient status.

Materials and methods

Culture conditions

Stock shoot cultures were grown in tissue culture containers (GA-7, Magenta Corp., Chicago, IL) with 40 ml MS medium mineral salts (Murashige and Skoog 1962), LS vitamins (Linsmaier and Skoog 1965) and 100 mg thiamine, 4.44 μM N^6-benzyladenine (PhytoTechnology Labs, Shawnee Mission, KS), 0.3 % agar (Phytotech A111) and 0.17 % Gelrite (Culture Gel Type I—Bio Tech Grade) at pH 5.7. Shoots were transferred to new medium every 3 weeks for two passage cycles and a total of 9 weeks of culture. At each passage, apical sections of shoots were removed and two node sections, (~10 mm), were transplanted to fresh medium with five shoots per container. Cultures were grown at 25 °C under a 16-h photoperiod with 70–90 μE m^{-2} s^{-1} irradiance provided by a combination of cool- and warm-white florescent bulbs.

Experimental design

Standard MS mesos include 2.99 mM $CaCl_2 \cdot 2H_2O$, 1.50 mM $MgSO_4 \cdot 7H_2O$ and 1.25 mM KH_2PO_4 (monobasic). Four concentrations (1.0, 1.5, 2.0, 2.5× MS) of the combined mesos components were tested with pear shoot cultures from the NCGR pear germplasm collection (Table 1). Five shoots of each of the 18 genotypes were planted on two GA-7 containers of each treatment medium ($n = 10$). Containers were assigned random numbers for arrangement on the growth room shelf. Each treatment group was transferred to fresh medium with the same mesos concentration at three week intervals for three passages ensuring complete stabilization on each treatment and shoots were evaluated after 9 weeks of growth.

Data

Factors evaluated were similar to those used for pear in earlier studies (Reed et al. 2013b) and included a subjective rating of overall shoot quality that includes both definable and non-definable characteristics (Niedz and Evens 2007). This gestalt rating scale provides a quick but accurate visual assessment of overall fitness of the plant for micropropagation; 1 poor (unacceptable for micropropagation), 2 acceptable, 3 good (the best plant for micropropagation). Quantitative data included shoot length (longest shoot measured in mm), shoot multiplication (shoots counted), leaf color (1 healthy green 2 yellow 3 red or brown), leaf spotting/necrosis (rated 1 absent, 2 minor, 3 major) and callus (1 absent, $2 \leq 3$, $3 > 3$ mm). Three plants at predetermined positions in each container were evaluated ($n = 6$) and the two remaining plants per container ($n = 4$) were photographed (non-subjective selection for data and images).

Statistical analysis

Experimental design, model evaluation, statistical analysis and graphical presentation were performed using Design

Table 1 Eighteen pear genotypes in six species evaluated for response to the mesos components of the growth medium

PI no	Plant ID	Genotype	Species
541013	662.001	Capital	*P. calleryana* Decne.
617561	2384.001	Ayers	*P. communis* L.
300693	38.001	Bartlett	
657931	2933.001	Horner 51	
541322	367.001	Luscious	
541415	1345.002	OH×F 87	
617654	2598.002	Pyrodwarf	
392323	585.001	Ubileen gift	
541285	1164.001	Winter Nelis	
541809	1660.001	NY 10353	*P. communis* × *P. ussuriensis*
541591	1589.001	*P. cordata*—Turkey	*P. cordata* Desv.
541829	815.001	*P. koehnei*	*P. koehnei* C. K. Schneider
541931	2149.002	Hosui	*P. pyrifolia* (Burm. F.) Nakai
541914	905.001	*P. pyrifolia* hybrid (Afghanistan seedling)	
541904	1173.002	Seuri Li	
289525	532.002	Sion Szu Mi	
315064	268.001	Hang Pa Li	*P. ussuriensis* Maxim.
542019	1318.001	Harbin	

PI no USDA Plant Introduction Number

Plant ID NCGR local identifying number

Expert 8 software (Design-Expert 2010). Data from six categories (shoot quality, shoot length, leaf spots, leaf color, and callus) were analyzed. Graphs representing data as simple trend lines of the interaction between combined mesos concentrations and 18 genotypes were presented. General linear model (GLM) procedure with analysis of variance (ANOVA) by SAS (9.2) program was also utilized for Dunnette's test contrasting between the mesos concentrations and the MS control for significance ($\alpha = 0.05$).

Quantitative ionic mineral analysis

Plant materials

Eight genotypes were selected and subcultured for comparisons of ion uptake on MS basal medium and the mesos concentration that was optimal for each. Four genotypes were compared on 1.0 and 1.5× mesos ('Hang Pa Li', 'Horner 51', *P. koehnei*, *P. pyrifolia*) and four others on 1.0 and 2.0× mesos ('Harbin', 'Old Home×Farmingdale 87' (OH×F 87), 'Sion Szu Mi', and *P. cordata*). Five shoots were planted in GA-7 containers with three replications ($n = 15$). Shoots were grown on each treatment for three passages for stabilization and harvested after 9 weeks growth. One shoot from a predetermined position in each container was selected and the three shoots were combined for the mineral analysis. Fresh weight was recorded before drying in the oven at 70 °C for 3 days, and transported to the analytical lab in Japan.

Sample preparation

Dried samples (0.05 mg), taken from three shoots cultured on each treatment were combined and placed into the muffle oven for 1 h at 500 °C to produce ash (total 24 samples). After cooling, 10 ml of 1 M HCl was added and the sample was completely dissolved. The supernatants were filtered through Whatman paper (No. 3) for purification. To measure ion concentrations in the samples, standard solutions (each of Ca, Mg, Fe, Na and K) were diluted as ion concentrations of 1.0, 0.5 and 0.01 ppm. An atomic absorption spectrometer (AAS) (Shimazu, Kyoto, Japan) and Ion Chromatography (IC) (Tohso, Tokyo, Japan) were employed for the quantitative ionic mineral analysis of the shoot samples. Analyses were performed in Department of Applied Chemistry and Biotechnology, Niihama National College of Technology in Japan.

Results

Seven of the nine *P. communis* genotypes showed significant improvements in overall shoot quality (Table 2) with increased mesos concentrations (Fig. 1a). 'Ayers' 'Bartlett' 'NY 10353' 'Pyrodwarf' and 'Ubileen Gift' had the best overall shoot quality on ≥2.0× mesos, while 'Horner 51', 'Luscious', and 'OH×F 87' had significantly increased shoot quality on ≥1.5× mesos (Table 3).

Mesos were less influential for shoot multiplication. Only 'Luscious' (2.0×) and 'Pyrodwarf' (1.5×) had

Table 2 Summary of the significance of six responses (P value <0.05) of 18 pear cultures as calculated by Design Expert

Genotype	Quality	Shoot number	Shoot length	Leaf spots	Leaf color	Callus
Ayers	<0.0001	NS	0.0101	NS	<0.0001	NS
Bartlett	0.0088	NS	0.0229	0.0003	0.0028	NS
Horner 51	0.0035	NS	0.0023	0.0095	NS	NS
Luscious	0.0010	0.0082	0.0155	<0.0001	<0.0001	0.0095
NY 10353	NS	NS	NS	NS	0.0082	NS
OH×F 87	<0.0001	NS	NS	0.0095	NS	NS
Pyrodwarf	0.0134	0.0003	NS	NS	NS	NS
Ubileen Gift	0.0002	NS	0.0007	0.0002	0.0010	0.0134
Winter Nelis	NS	NS	NS	NS	NS	NS
Hosui	NS	NS	NS	0.0058	0.0002	NS
P. pyrifolia hybrid	0.0041	NS	NS	0.0063	NS	0.0006
Seuri Li	NS	NS	NS	NS	0.0289	NS
Sion Szu Mi	0.0187	NS	NS	0.0058	0.0088	NS
Hang Pa Li	0.0172	NS	NS	NS	NS	NS
Harbin	NS	NS	0.0006	NS	NS	NS
P. cordata	NS	NS	0.0061	NS	0.0083	NS
P. koehnei	NS	NS	NS	NS	NS	0.0221
Capital	NS	NS	0.0238	NS	NS	NS

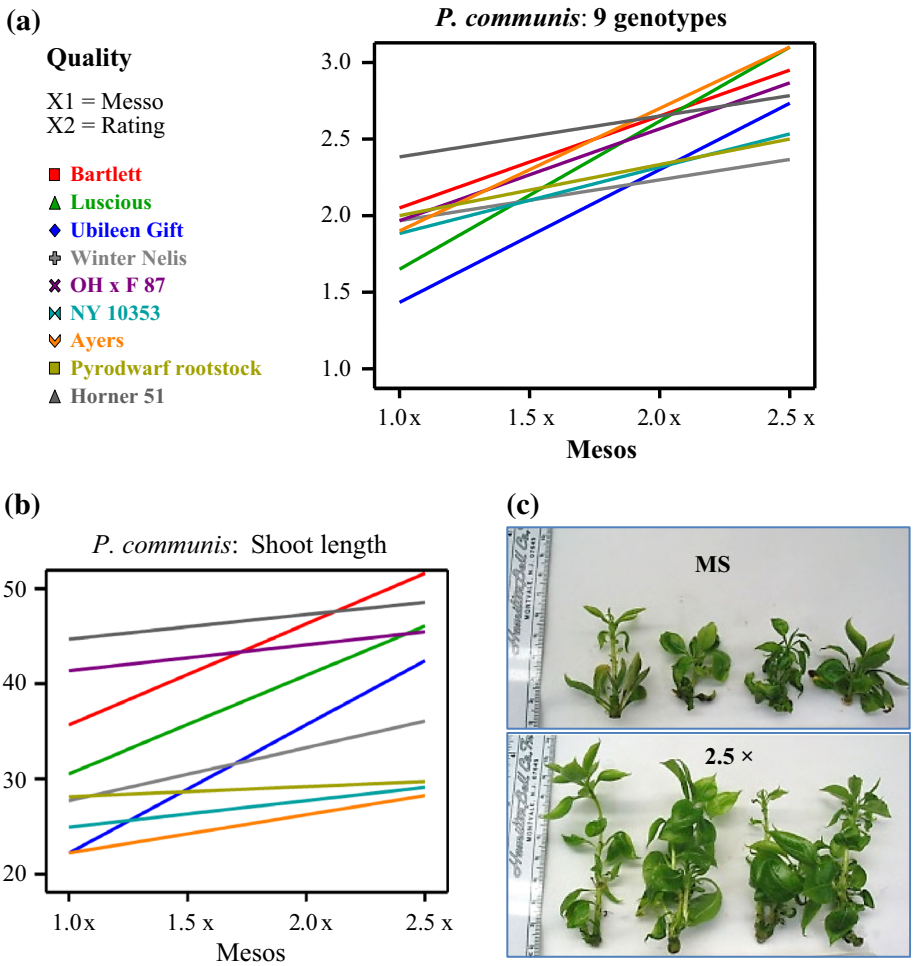

Fig. 1 Trend lines of the interaction graph for shoot quality (ratings: 1 poor, 2 moderate, 3 good) (**a**) and shoot length (mm) (**b**) of nine *P. communis* genotypes. **c** Photos: 'Bartlett' shoots cultured on MS and high mesos medium

significantly increased multiplication with an increase in mesos (Tables 2, 3 and ESM 1). 'Luscious' produced the best multiplication on 2.0× medium with a mean of 4.5 shoots (1.67 shoots in MS), while 'Pyrodwarf' on 1.5× medium produced a mean of 4.33 shoots (2.17 shoots in MS). Shoots were significantly longer (Table 2) for five genotypes grown on higher mesos concentrations: 'Ayers' (≥2.0×), 'Bartlett' (≥1.5×), 'Horner 51' (1.5×), 'Luscious' (≥1.5×), and 'Ubileen Gift' (≥2.0×) (Table 3; Fig. 1b, c).

Leaf spots were significantly reduced for five genotypes (Table 2) and eliminated for all on increased mesos (Table 3 and ESM 1). Five cultivars had significantly greener leaves (Table 2) with increased mesos (ESM 1). All *P. communis* cultivars produced low amounts of callus (ESM 1). The only genotypes with noticeable callus were 'Luscious' and 'Ubileen Gift' and there was less callus on high mesos treatments.

Shoot quality significantly improved with increasing mesos concentrations for two *P. pyrifolia* genotypes (Table 2) and both had good shoot quality on 2.5× (Table 3; Fig. 2a). Shoot number, shoot length and leaf size were not significantly improved by increased mesos for any of the four genotypes (Table 2; Fig. 2b and ESM 2). Increasing mesos to ≥1.5× significantly reduced leaf spots for two accessions and was effective at 2.0× for a third (ESM 2). Leaf color was best at 2.0–2.5× for three genotypes (Table 3). Callus was initially low, but decreased significantly with increasing mesos for the *P. pyrifolia* hybrid (ESM 2).

The best quality shoots for *P. ussuriensis* 'Hang Pa Li' were produced on 1.5× mesos medium and shoot quality greatly decreased at higher concentrations (Tables 2, 3). This was the only genotype that had lower shoot quality at the higher mesos concentrations (Fig. 3a). The other genotypes in this group had no significant changes in shoot quality over the range of mesos concentrations (Table 2).

Shoot length significantly increased for *P. cordata*, *P. ussuriensis* 'Harbin', and *P. calleryana* 'Capital' (Tables 2, 3). Taller shoots were seen when the mesos increased to ≥2.0× except for 'Hang Pa Li' (Fig. 3b, c). Leaf color improvements with increased mesos were only significant for *P. cordata* (Tables 2, 3 and ESM 3). Meso factors were not significant for shoot number or leaf spots for any of the five genotypes (Table 2). *P. koehnei* produced a large amount of callus on MS but this was significantly reduced as mesos increased to 2.0 or 2.5× (Tables 2, 3 and ESM 3).

Quantitative ionic analysis

Shoots of five of the eight genotypes grown for nutrient analysis had significantly increased fresh weight ($P \leq 0.05$) when compared those grown on the control (MS level: 1.0×) medium (Table 4). Analysis by ion spectrophotometry revealed that Ca, Mg, Na, and K were all at higher ionic concentrations (ppm) in the shoot samples cultured in high mesos compared to the control while iron (Fe) was lower (Figs. 4, 5). Fe was reduced by 27 % (*P. koehnei* and *P. pyrifolia*) to 49 % ('Horner 51') on

Table 3 The mesos concentrations that produced significantly higher means than the MS control for 18 pear genotypes

Genotype	Quality	Shoot number	Shoot length	Leaf spots	Leaf color	Callus
Ayers	2.0/2.5	NS	2.0/2.5	NS	2.0/2.5	NS
Bartlett	2.0/2.5	NS	≤1.5	≤1.5	≤1.5	NS
Horner 51	1.5/2.5	NS	1.5	≤1.5	NS	NS
Luscious	≤1.5	2.0	≤1.5	≤1.5	≤1.5	≤1.5
NY 10353	2.5	NS	NS	NS	2.5	NS
OH×F 87	≤1.5	NS	1.5	≤1.5	NS	NS
Pyrodwarf	2.5	1.5	NS	NS	NS	NS
Ubileen Gift	2.0/2.5	NS	2.5	2.5	2.5	2.0/2.5
Winter Nelis	NS	NS	2.0	NS	NS	NS
Hosui	NS	NS	NS	≤1.5	2.5	NS
P. pyrifolia hybrid	1.5/2.5	NS	1.5	≤1.5	NS	≤1.5
Seuri Li	NS	NS	1.5	NS	2.0	NS
Sion Szu Mi	2.5	NS	NS	2.0/2.5	2.5	NS
Hang Pa Li	1.5	NS	NS	NS	NS	NS
Harbin	NS	NS	≤1.5	NS	NS	NS
P. cordata	NS	NS	1.5/2.5	NS	1.5	NS
P. koehnei	NS	NS	NS	NS	NS	2.0/2.5
Capital	NS	NS	2.5	NS	2.5	NS

Means compared to control (MS mesos) calculated by Dunnette's test

NS = not significantly different at $P \leq 0.05$

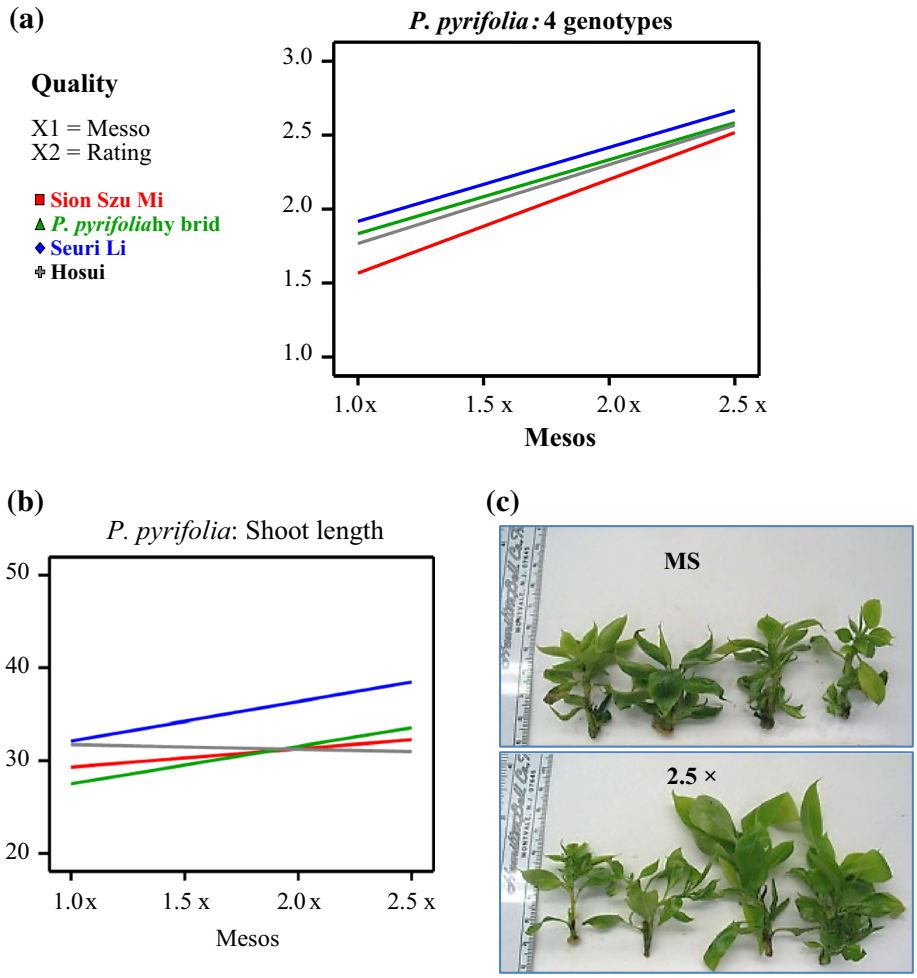

Fig. 2 Trend lines of the interaction graph for shoot quality (ratings: 1 poor, 2 moderate, 3 good) (**a**) and shoot length (mm) (**b**) of four *P. pyrifolia* genotypes. **c** Photos: 'Sion Szu Mi' shoots cultured on MS and a high mesos medium

1.5× mesos; reductions were also seen on 2.0× mesos with a range of 46 % (*P. cordata*) to 60 % ('Harbin') (Fig. 5).

Discussion

A diverse response was expected from these pear accessions due to the wide range of genetic variability found in gene banks. This study of 18 pears in six species displays some of that diversity, but also some unifying factors. In this study the mesos components were varied relative to the concentrations in MS and in most cases increased concentrations were effective for increasing the growth and quality of many of these diverse pear shoots. Testing a range of mesos concentrations provided a simple way to characterize this set of diverse pear germplasm and to determine changes that would improve a general pear growth medium. The genotypes tested belonged to six species (Table 1) and all of them had significant responses to increased mesos for at least some of the evaluated categories. There was significant improvement in shoot quality for 10 genotypes, mostly *P. communis* and *P. pyrifolia* cultivars. The visual quality factor was the best way to evaluate the best propagules for micropropagation. This was earlier shown to be an effective rating tool that could be analyzed statistically to differentiate among treatments (Niedz et al. 2007). Shoot length, leaf spots and leaf color were the factors most affected by the mesos components and that greatly impacted the quality ratings of the shoots. A few accessions had improved shoot numbers or less callus with increased mesos concentrations, however, shoot multiplication and callus for most pears responded best to changes in the nitrogen components (Wada et al. 2014 #3916; Reed et al. 2013a #2849). A prior study that examined each of the three mesos components as separate factors found that eight of the nine genotypes tested required ≥5× Ca and Mg and five of nine required higher concentrations of all three minerals (Wada et al. 2013). In several cases the individual components tested in the first study were not significant for shoot quality for some cultivars ('Ayers', 'Horner 51'), but when all three were increased simultaneously in the present study the

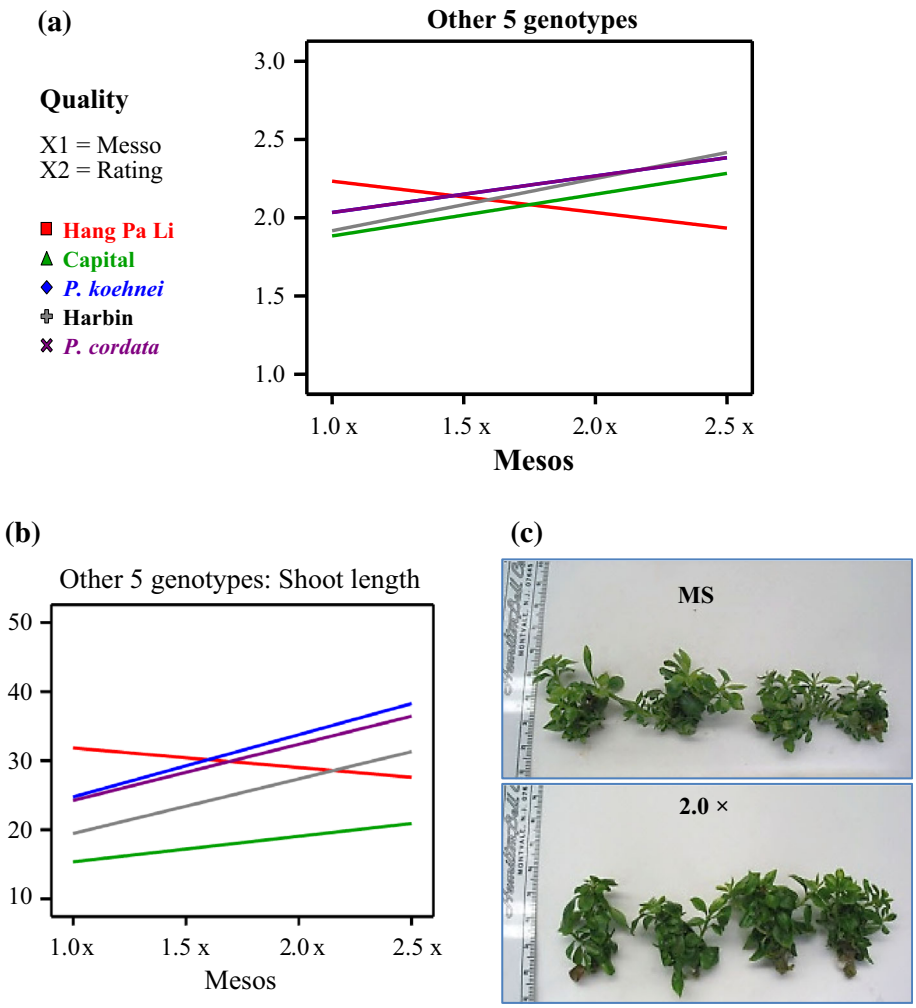

Fig. 3 Trend lines of the interaction graph for shoot quality (ratings: 1 poor, 2 moderate, 3 good) (**a**) and shoot length (mm) (**b**) of *P. koehnei*, *P. calleryana* 'Capital', *P. cordata, P. ussuriensis* 'Hang Pa Li' and 'Harbin'. **c** Photos: *P. cordata* shoots cultured on MS and high mesos medium

Table 4 Mean fresh weight of pear cultures grown on MS medium or with 1.5 or 2.0× mesos

Pear name	Meso conc. (MS: 1.0×)	MS medium	Increased mesos	*t* test	
		Fresh weight (g) ± SE	Fresh weight (g) ± SE	*P* value	Significance
Hang Pa Li	1.0/1.5	0.455 ± 0.08	0.730 ± 0.11	0.043	*
Horner 51		0.447 ± 0.04	0.742 ± 0.07	0.009	**
P. koehnei		0.866 ± 0.17	1.497 ± 0.09	0.084	ns
P. pyrifolia		0.764 ± 0.13	1.313 ± 0.06	0.008	**
Harbin	1.0/2.0	0.574 ± 0.09	1.489 ± 0.18	0.029	*
OH×F 87		0.381 ± 0.03	0.634 ± 0.09	0.062	ns
Sion Szu Mi		0.553 ± 0.07	1.354 ± 0.21	0.057	ns
P. cordata		0.306 ± 0.12	0.850 ± 0.12	0.025	*

SE = standard error calculated at $P = 0.05$

ns = not significantly different at $P \leq 0.05$

* Significantly different at $P \leq 0.05$; ** significantly different at $P \leq 0.01$

results were significant (Table 2). In other cases only one or two of the individual components were significant in the first study, but increasing all of them at once also produced improved shoot quality ('Luscious', 'OH×F 87', 'Sion Szu Mi'). The quality of other cultivars improved for individual mesos components, but was not significant when all were

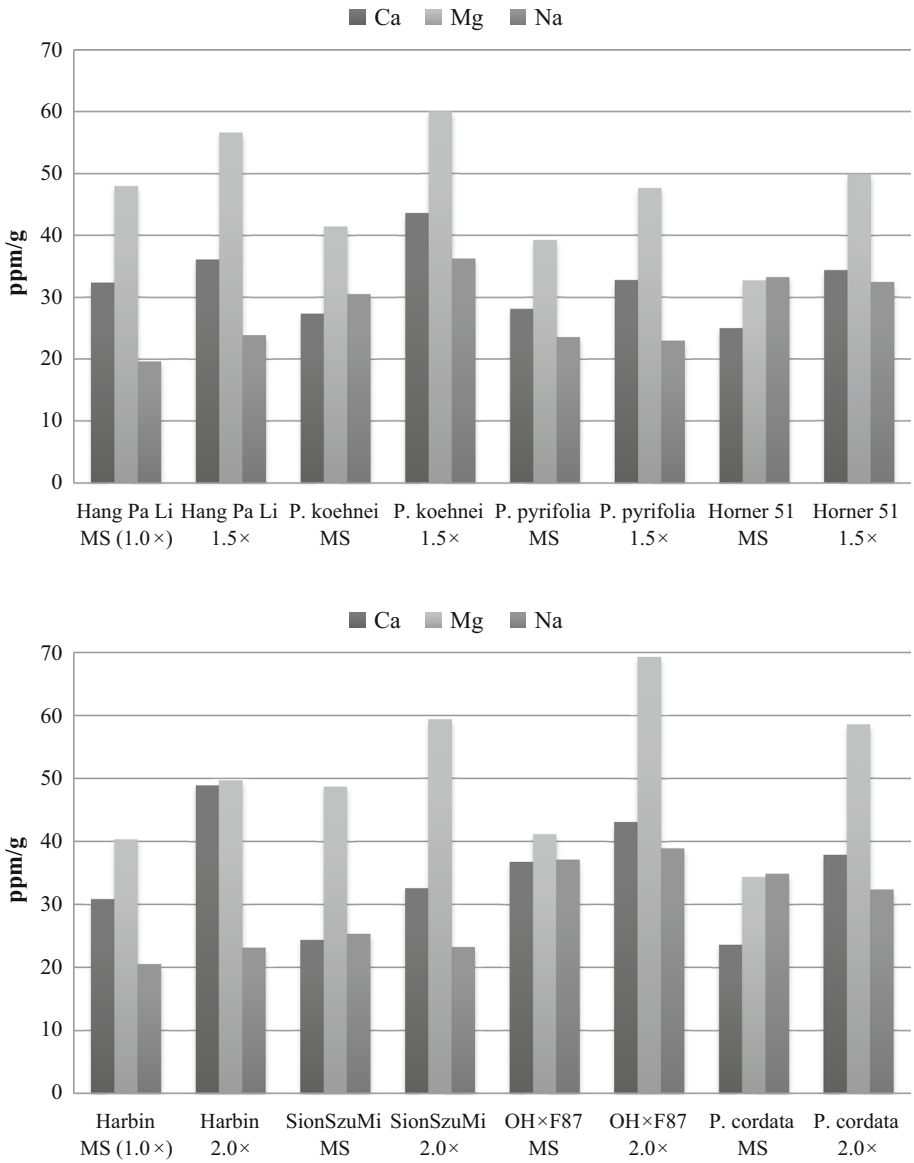

Fig. 4 Ca, Mg, Na ion composition of shoots cultured on MS control or increased mesos (1.5 or 2.0×) concentrations

increased ('Capital', 'Winter Nelis', *P. koehnei*). The differences may be because the first study was based on modeling the plant responses while the current study directly tested the range of concentrations. In addition, the interaction of mineral nutrients for both uptake and metabolism in plants is a likely factor in these responses (Marschner 1995; Troyanos et al. 2000). The initial mesos study (Wada et al. 2013) found that the individual meso factors were not highly influential for shoot multiplication for most genotypes and that was evident with these genotypes as well. Significant improvement in shoot multiplication for several of these genotypes required changes in the MS nitrogen components (Wada et al. 2014).

Several interesting results of ion analysis were evident from this study. As expected, Ca, Mg and K were present in higher amounts in plants from the 1.5 or 2.0× treatments compared to the control (Figs. 4, 5). Significantly more biomass was produced by five of the eight cultivars grown on the higher mesos concentrations compared to the MS level (Table 4). This indicated that the MS concentration of mesos was limiting growth for most of the pears evaluated. Pear shoots cultured on the 1.5 or 2.0× mesos media were green with no chlorosis or leaf spots, and all had less Fe (27–60 % reduction) compared to the shoots cultured in MS (Fig. 5). Higher mesos, especially Mg, contribute to increased chlorophyll content (Marschner 1995; Wada et al. 2013). We did not alter the amount of Fe in the medium in this study, so it may be that Fe uptake was inhibited by increased phosphate in the medium (Fig. 5). More Fe may not necessarily contribute to leaf color if it is already available in moderate amounts.

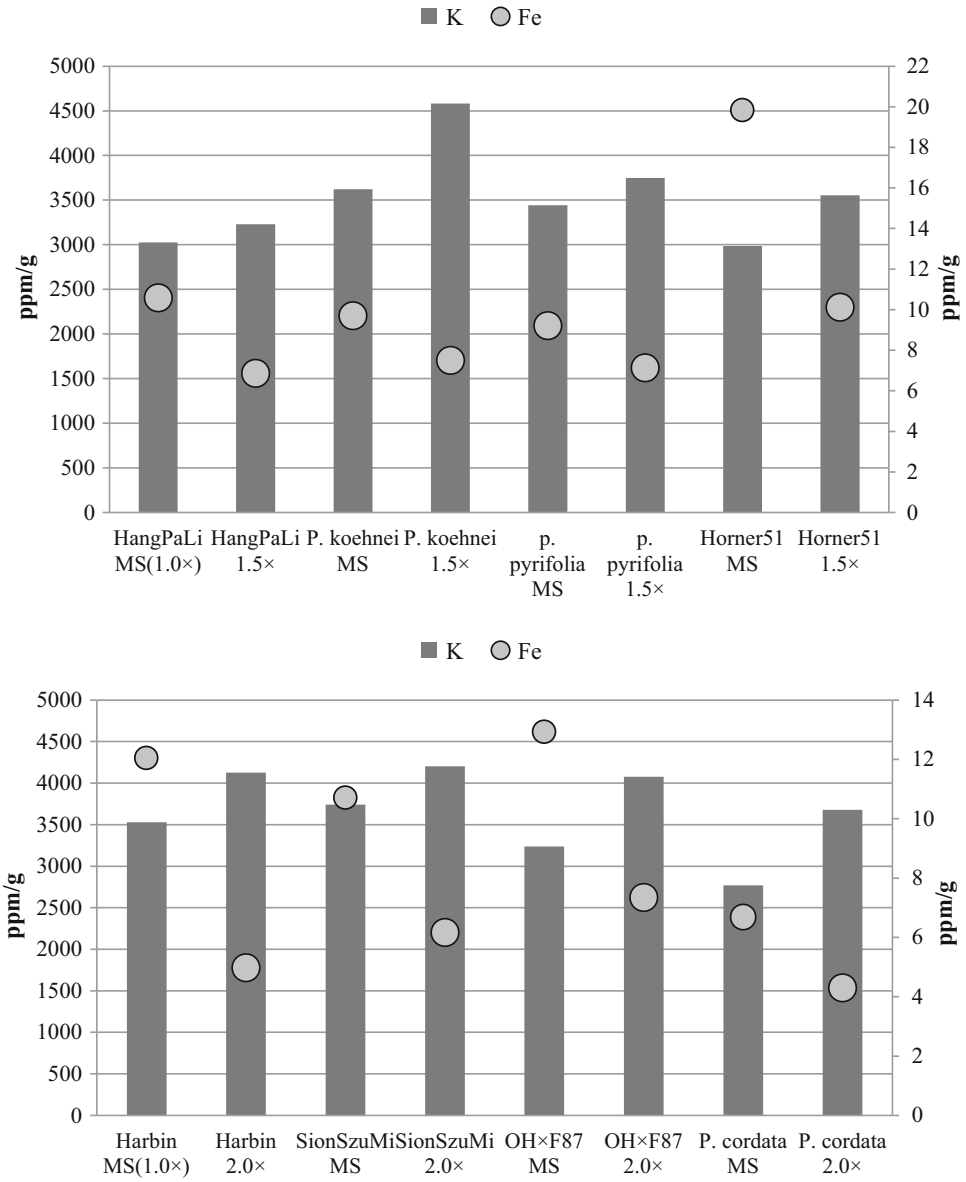

Fig. 5 K and Fe ion composition of shoots cultured on MS control or increased mesos (1.5 or 2.0×) concentrations

Most studies of medium alterations test only one or two genotypes or only look at growth regulator responses. Laboratories that work with large numbers of diverse genotypes are not able to develop or provide optimal growth media for all, but aim to find a suitable medium to produce moderate growth. Simplifying medium preparation by making changes that improve the growth of most, if not all genotypes, would improve the number of genotypes that can be commercially propagated, held in germplasm collections or used for cryopreservation. Altering all of the meso components at the same time proved generally effective for all 18 genotypes and was not detrimental to any. It was especially effective for the *P. communis* and *P. pyrifolia* cultivars with improvements in shoot quality for most genotypes. The approach was also positive for the other four species, but with fewer significant improvements for each (Tables 2, 3). After evaluation, the 18 genotypes were divided into groups based on their meso requirements and grown on either 1.5 or 2.0× concentrations. All of the problematic-, difficult-, and slow-growing pear germplasm from around the world maintained in the USDA-ARS National Clonal Germplasm Repository (NCGR) can now be cultured using these increased mesos concentrations and for some, alterations in the nitrogen components.

Authors' contribution statement Dr. Sugae Wada planned and performed the experiments, data collection, analysis and manuscript preparation. Dr. Shinya Maki provided the ionic analysis. Dr. Randal Niedz provided assistance with experimental design, statistical analysis and

manuscript review. Dr. Barbara Reed was responsible for planning and supervising all experiments, assisting with data analysis, manuscript preparation and manuscript review.

Acknowledgments We thank NCGR lab personnel for assistance with collection of the data. This project was funded by a grant from the Oregon Association of Nurseries and the Oregon Department of Agriculture and by USDA-ARS CRIS project 5358-21000-0-38-00D.

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