MICROPROPAGATION

Relationships between nutrients and plant density in liquid media during micropropagation and acclimatization of turmeric

Jeffrey Adelberg · Todd Driesse · Sean Halloran · William C. Bridges

Abstract To improve micropropagation and acclimatization of turmeric (*Curcuma longa* L.), we evaluated the effects of media volume, plant density, macronutrient ion concentrations, cationic ratios $[NH_4^+]/[K^+]$, and sucrose concentration. Multiplication was highest with low bud density. Yield of new plants was highest with high bud density, the most sucrose, the highest concentration of macronutrients, and the greatest volume of medium. However, maximum plant size required low-density, reduced sucrose and elimination of NH_4^+. The largest plants grew quickest during greenhouse acclimatization when macronutrients were lowered to 20 mM. In a follow-up experiment, media volume was set at 40 mL with 5% sucrose with NH_4^+ reduced to 5 mM, and the effects of varying P, Ca, Mg, KNO_3, and bud densities were assessed. The largest plants were produced at low density. More importantly, at high density the optimal concentrations of P, Ca, Mg, and KNO_3 predicted plant size that was nearly equal to the maximum value from low-density cultures in the prior experiment. Growth of plants during greenhouse acclimatization was increased by modifications of *in vitro* medium with plants cultured with 3.32 mM P, 4.5 mM Mg, and 37 mM KNO_3 predicted to grow most rapidly. The effect of starter fertilizer in the greenhouse mix was much less than the effects of P, Mg, and KNO_3 *in vitro*. These results showed (1) optimal media formulae for different stages of micropropagation and (2) process-related factors such as plant density and media volume affected the optimal nutrient concentrations.

Keywords *Curcuma longa* · d-Optimal · Mineral nutrition · Multifactor · Response surface model

Introduction

Micropropagation practices using tissue culture results in the production of hundreds of millions of plants annually. About 80% of reported micropropagation protocols use MS (Murashige and Skoog 1962) medium (George and De Klerk 2009). Murashige and Skoog developed this medium through maximizing tobacco callus growth by altering nutrients one factor at a time (OFAT) in semi-solid agar medium with 3% sucrose. It might be useful to develop additional media formulations for micropropagation because shoot buds and tobacco callus likely have different nutritional requirements for optimal responses (Ramage and Williams 2002). There is a general lack of information on correct nutrition for developing shoots prior to greenhouse culture.

Media have also been developed for soybean (Gamborg et al. 1968; Schenk and Hilderbrandt 1972), woody shrubs (Anderson 1980; Lloyd and McCown 1980), nut and hardwood trees (Driver and Kuniyuku 1984), epiphytic orchids (Knudson 1946; Vacin and Went 1949), protoplasts (Kao and Michayluk 1975), and anthers and microspores (Nitsch and Nitsch 1969; Chu et al. 1975). These media formulations were generated using OFAT without the benefit of more recently developed software tools for experimental design, computation, and graphical visualization.

Combinations of nutrients and other process factors can be arranged in geometric, multiple factor designs involving relatively few samples to allow simultaneous effects to be

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evaluated on varied plant responses. Nutrient ions, instead of salts, may be analyzed if counter ion confounding is correctly considered in the design (Niedz and Evens 2006). Response surface models, limited to second-order interactions, have been used to identify critical factors driving callus and regeneration (Niedz and Evens 2007; Niedz et al 2007). When comparing ion concentrations in plant tissue culture basal salts (Owen and Miller 1992), nitrate is most often required in larger concentrations than cationic nutrients, yet charge balance constrains ion optimization designs. In one such design, the equivalence of NO_3^- was set to equal the combined equivalence of the macronutrient cations, NH_4^+, and K^+ (Niedz and Evens 2006). Multifactor designs with response surface models were also used to effectively screen nutrient salt components of MS for micropropagation of pear in two tandem experiments (Reed et al 2013; Wada et al 2013). The first design clustered mineral nutrients in MS medium into five groups (NH_4NO_3, KNO_3, mesonutrients, micronutrients, and iron) and showed that lowered concentrations of NH_4NO_3 supported greater numbers of shoots. The second design showed that increased concentrations of mesonutrient salts ($CaCl_2$, $MgSO_4$, and KH_2PO_4) improved shoot quality. Several difficult-to-micropropagate genotypes had improved responses with altered mineral nutrition.

In commercial micropropagation, plants are often grown at high densities to get as many plants as possible from a vessel. Spent medium analysis (determining the amount of nutrient left in the medium following a culture period) shows specific elements that limit growth, particularly in high-density, liquid cultures (Leifert et al. 1995). Deficiencies or imbalances in MS were identified by apportioning the amount of nutrients used to the plants mass and comparing results with published agronomic data for a particular crop (Adelberg et al. 2010). These imbalances negatively impact growth in high-density cultures when plants with little or no roots are transferred from the nutrient-rich, enclosed laboratory environment to the greenhouse mist system, where nutrients are leached from an open system.

Turmeric (*Curcuma longa* L.) is an important culinary and medicinal herb native to South and South-East Asia with a variety of secondary metabolites of interest to modern and traditional medicine (Sarkar and Nahar 2007). Turmeric has been a model for micropropagation of other herbaceous perennial monocots such as gingers, bananas, taro, lilies, bamboo, and many of the ornamental geophytes (Adelberg 2010). Media alterations for turmeric tissue culture involved slight modification of MS salts (Nirmal-Babu et al. 2007). With a model clone of turmeric (L35-1), macronutrients were altered in a micronutrient-limited environment to observe how plant mineral nutrition *in vitro* impacted growth in the greenhouse (Halloran and Adelberg 2011). As a result of these imposed nutrient limitations, lowered plant density in culture resulted in plants that grew better in the greenhouse.

Understanding the relationships between nutrient concentrations and plant density can help identify media formulations that eliminate nutrient deficiencies. This allows optimization of growth at high plant densities during micropropagation and subsequent acclimatization. Two multifactor experiments were conducted sequentially to systematically screen essential concentrations of nutrients while maximizing plant density. Factors in the first experiment included the number of plants, volume of media/vessel, and sucrose and macronutrient concentrations. Spent medium was analyzed to observe nutrient limitations during rapid growth. Results from the first experiment identified the volume of medium, ammonium, and sucrose concentrations that were fixed in the second experiment. In the second experiment, multiplication and growth during micropropagation and greenhouse acclimatization were maximized using KNO_3 and mesonutrients at various plant densities. Growth in the greenhouse, with and without starter fertilizer, was used to assess the effects of nutrients application *in vitro* on subsequent growth in greenhouse compared to nutrients provided in the greenhouse soilless mix.

Materials and Methods

Plant material. Stage I explants of *Curcuma longa* L. accession 35-1 were initiated according to Cousins and Adelberg (2008). Accession L35-1 was obtained from University of Arizona Southwest Center for Natural Products Research and Commercialization (UA Herbarium 375,742, ARIZ), and prepared via dissection of quiescent shoot tips from rhizomes. Shoot tips were disinfested in full-strength commercial bleach (Clorox™, 5.25% sodium-hypochlorite) for 30 s, placed on the hood surface to dry, and finally transferred to MS medium modified with 170 mg NaH_2PO_4, 100 mg myo-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine hydrochloride per liter, and 3% sucrose. Medium pH was adjusted to 5.7 with 1 N KOH and explants were propagated at stage I using 2 mL of liquid medium in flat-bottomed vials (O.D.×H.: 29×94 mm; Fisher glass shell vials, Thermo Fisher Scientific Inc., Pittsburgh, PA). Stage II plants obtained from these cultures were maintained for approximately 5 year by subculture in the medium above (except with 6% sucrose and 1 μM benzyladenine) in 33 mL of medium in a cylindrical glass jar (180 ml) with a Magenta B cap closure (Magenta Corp., Chicago, IL) on a 100 r.p.m. orbital shaker, at 23±2°C under cool white fluorescent lights with the intensity of 25–30 μmol m²s⁻¹ photosynthetically active radiant (PAR) for 16 h/d. Immediately prior to experiments, stage II plants were multiplied on media containing 3 μM benzyladenine and 3% sucrose.

In vitro experiments. In the first experiment, process factors altered included media volume (25, 35, 45 mL/vessel), bud

density (three, six, nine buds/vessel), and nutritive factors such as sucrose concentration (1.5%, 3.75%, 6%), macronutrient ion concentration (with 10, 30, 50 mM NO_3^-), with equivalence balancing cations with $[NO_3^-]$, and NH_4^+/K^+ ratio (0:1, 0.125:0.875, 0.25:0.75, 0.5:0.5). Fifty different treatment combinations (with five true replicates) were chosen using the d-optimal criterion as described by Halloran and Adelberg (2011; Electronic supplemental materials A). The design constrained the sum of ammonium and potassium (the predominant cations) to equal nitrate concentration (the predominant anion) to eliminate pH and counter ion confounding of nutritive effects (Niedz and Evens 2006). All other nutrients were supplied at 100% of MS salt concentrations (Murashige and Skoog 1962).

In the second experiment, media were prepared with 5% sucrose and 5 mM ammonium, adjusted to a pH of 5.8 using 1 N KOH, and dispensed at 40 mL per vessel. All nutrients were supplied at MS levels except for the mesonutrients P (1.25, 3.75, 6.25 mM), Ca (3, 6, 9 mM), Mg (1.5, 3, 4.5 mM), and the macronutrients KNO_3 (20, 40, 60 mM). Plant density was also varied (three, six, nine buds/vessel), and these factors were arranged into 32 different treatment combinations (with two true replicates) chosen using the d-optimal criterion (Electronic supplemental materials B). To maintain charge balance with varying concentrations of Ca, Mg, and KH_2PO_4, the sulfate concentration was varied from 4.1 to 16.5 mM, confounding the treatment effects.

In both experiments, plants were assigned to treatment media and grown for three consecutive 35-d cycles. During subculture, plants were counted and weighed, spent media volume was determined, and sucrose remaining in the media was measured on a refractometer (Atago Model N10, Atago

Instruments Ltd., Toyko, Japan). After the third *in vitro* cycle, a representative tissue sample was taken from each treatment for dry matter analysis. Plant material for dry mass was dried for 72 h at 75°C (relative dry mass=dry/fresh mass). Laboratory responses were analyzed from two replicates of each cycle 3 vessel. Spent medium was frozen for analysis at the conclusion of cycle 3.

Greenhouse experiments. After the third *in vitro* cycle, the tissue (excluding the dry matter analysis sample) was moved to a greenhouse mist bed in Fafard 2-B (without starter

Table 1 Multiplication rate of turmeric following three 35-d cycles was modeled with terms that included: combined equivalence of three macronutrient ions (NH_4^+, K^+, NO_3^-), the proportion of NH_4^+/K^+, media volume, numbers of buds/vessel, and initial sucrose concentration. The final model had $R^2=0.448$, $R^2_a=0.306$, $R^2_p=0.0869$, overall model F ratio was 3.167 ($P<0.0032$), and AICc was 155.327

| Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	−0.1097±0.0420	0.0123	4.5726
Volume×sucrose	0.0144±0.0057	0.0148	4.3109
Macronutrients×volume	0.0008±0.0004	0.0289	3.4182
Buds/vessel×sucrose	−0.0324±0.0196	0.1060	1.8238
Volume	0.0205±0.0128	0.1164	1.7176
Volume×volume	−0.0050±0.0031	0.1169	1.7132
NH_4/K ratio×volume	0.0933±0.0601	0.1277	1.6141
Sucrose	0.0776±0.0543	0.1604	1.3652
NH_4/K ratio	0.7181±0.5499	0.1985	1.1413
NH_4/K ratio×NH_4/K ratio	4.7514±4.0366	0.2456	0.9271
Macronutrients	0.0029±0.0033	0.3818	0.5224

[a] P value of T test
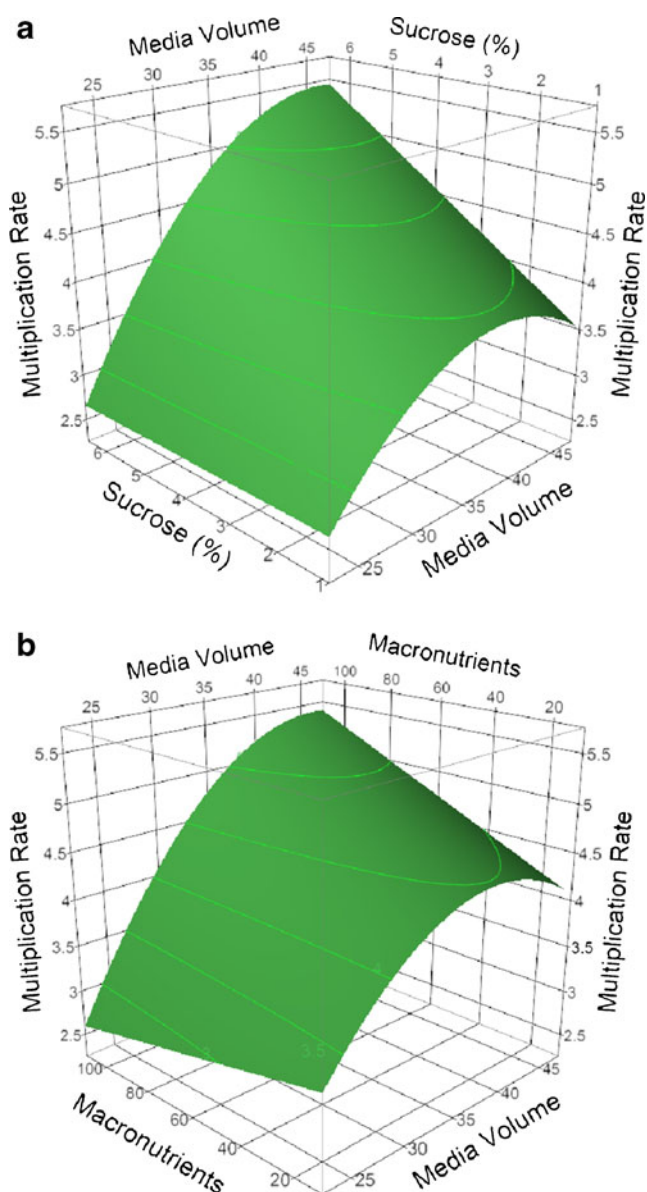
Figure 1. Response surface plot illustrates subsets of the multiplication ratio model in experiment 1: (*A*) media volume×sucrose with NH_4/K ratio set at 0.5, macronutrients at 100 mM, and buds/vessel at three; (*B*) media volume×macronutrients with NH_4/K ratio set at 0.5, buds/vessel at three, and sucrose at 6%.

fertilizer in the first experiment, and with or without starter fertilizer in the second experiment) soilless mix (Canadian sphagnum peat moss, 3/8″ processed pine bark, perlite, vermiculite, wetting agent, and dolomitic limestone; Fafard Co., Anderson, SC). In an effort to keep a relatively uniform planting density, plants from vessels with three initial buds were planted in one 601 cell ($16 \times 12 \times 5.5$ cm polyethylene insert, Landmark Plastics, Akron, OH). If six buds were used, they were acclimatized in two 601 cells, and if there were nine buds, three 601 cells were used. After 14 d in the greenhouse (latitude=34.67350, longitude=−82.83261; 60% shade cloth; mist cycle was 6 s on every 16 min during daylight h), plants were harvested, roots were washed clean of soil, weighed again, and dried. The difference between plant mass going into the greenhouse and plant mass after 14 d in the mist bed was recorded. Survival of plants was reported as percentage of plants per vessel that survived greenhouse acclimatization.

Calculated responses. Direct observations from the laboratory and greenhouse allowed calculated responses as follows:

$$Multiplication\ rate = \left(\frac{Plants\ out\ per\ vessel}{Buds\ in\ per\ vessel}\right)$$ was used to determine the relative level of multiplication in a fixed time period (35-d cycle).

Number of new plants per vessel= Plants out per vessel− Buds in per vessel was the measure of the productive output of each vessel *in vitro*.

Fresh weight per plant= Total fresh weight/Number of plants per vessel was a measure of the weight of the plants *in vitro*.

Table 2 New plants per vessel of turmeric following three 35-d cycles was modeled with terms that included: combined equivalence of three macronutrient ions (NH_4^+, K^+, NO_3^-), the proportion of NH_4^+/K^+, media volume, numbers of buds/vessel, and initial sucrose concentration. The final model had R^2=0.671, R^2_a=0.587, R^2_p=0.4482, overall model F ratio was 7.969 ($P<0.0001$), and AICc was 331.146

Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	1.5620±0.2072	<0.0001	929.2018
Volume×sucrose	0.0800±0.0281	0.0067	132.8924
Macronutrients×volume	0.0045±0.0017	0.0131	109.6767
Volume×volume	−0.0320±0.0152	0.0412	72.4893
Macronutrients	0.0251±0.0163	0.1312	38.7481
NH_4/K ratio×NH_4/K ratio	27.2737±20.1331	0.1826	30.0255
NH_4/K ratio	3.6554±2.7244	0.1867	29.4546
Volume	0.0789±0.0633	0.2192	25.4416
NH_4/K ratio×volume	0.3686±0.2968	0.2209	25.2411
Macronutrients×sucrose	0.0092±0.0076	0.2308	24.1736
Sucrose	0.1941±0.2687	0.4740	8.5384

[a] P value of T test

Survival in the green house $= \left(\frac{Plants\ day\ 14}{Plants\ day\ 0}\right)$, calculated as the number of plants in the greenhouse on day 14 (per vessel) divided by plants brought to greenhouse at day 0 (per vessel).

Greenhouse relative growth ratio= Fresh weight per plant after 14 d in greenhouse/Fresh weight per plant at day 0 in greenhouse used as a measure of how much the plants grew once moved to greenhouse conditions.

Spent medium analysis. Residual media from cycle 3 was analyzed by Clemson University's Agricultural Chemical
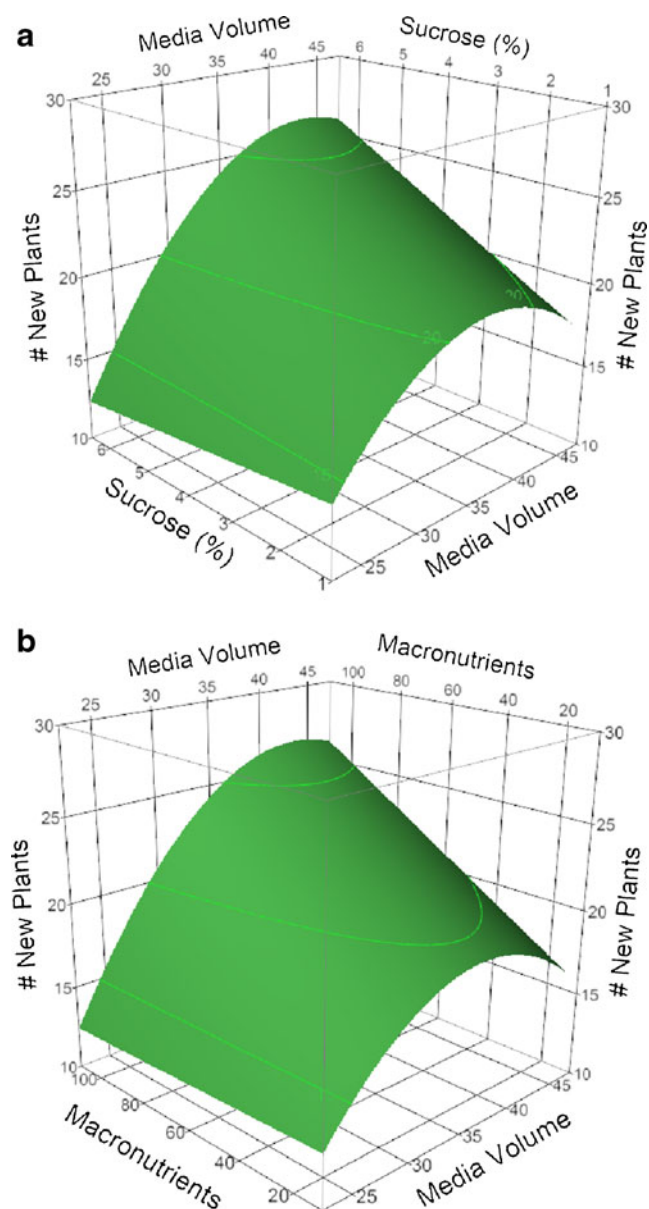

Figure 2. Response surface plot illustrates subsets of the new plants/vessel model in experiment 1: (*A*) media volume×sucrose with NH_4/K ratio set at 0.5, macronutrients at 100 mM, and buds/vessel at nine; (*B*) media volume×macronutrients with NH_4/K ratio set at 0.5, buds/vessel at nine, and sucrose at 6%.

Table 3 Plant size of turmeric following three 35-d cycles was modeled with terms that included: combined equivalence of three macronutrient ions (NH_4^+, K^+, NO_3^-), the proportion of NH_4^+/K^+, media volume, numbers of buds/vessel, and initial sucrose concentration. The final model had $R^2=0.712$, $R^2_a=0.620$, $R^2_p=0.4124$, overall model F ratio was 7.784 ($P<0.0001$), and AICc was 7.097

Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	-0.0531 ± 0.0105	<0.0001	1.0674
Macronutrients	0.0042 ± 0.0009	<0.0001	0.9120
Volume	0.0138 ± 0.0031	<0.0001	0.8259
Sucrose	-0.0373 ± 0.0135	0.0085	0.3165
NH_4/K ratio	-0.2877 ± 0.1363	0.0410	0.1844
Volume×sucrose	0.0030 ± 0.0014	0.0419	0.1826
Macronutrient×macronutrients	-0.0001 ± 0.00004	0.0425	0.1815
NH_4/K ratio×sucrose	0.1353 ± 0.0670	0.0502	0.1686
Buds/vessel×sucrose	0.0088 ± 0.0048	0.0763	0.01369
NH_4/K ratio×macronutrients	-0.0066 ± 0.0043	0.1279	0.1000
NH_4/K ratio×volume	-0.0225 ± 0.0151	0.1435	0.0921
Macronutrients×buds/vessel	-0.0003 ± 0.0003	0.2471	0.0571
Volume×buds/vessel	-0.0013 ± 0.0011	0.2472	0.0571

[a] P value of T test

Service using a Thermo Jarrell Ash Model 61E Inductively Coupled Plasma (ICP; Analytical West, Inc., Corona, CA). The amount of nutrient residual was calculated as the ICP-determined concentration multiplied by volume of spent medium for the elements P, Ca, Mg, Zn, Cu, Mn, Fe, S, Na, B, and Cl. Use was expressed as a percentage of total supplied. The concentrations of these nutrients in tissue were calculated and expressed as mg nutrient used per kilogram tissue dry mass.

Statistical design and analysis of the experiments. Response surface models were proposed to relate the five factors to growth responses. The models included linear, quadratic, and interaction terms of the factors. The first experiment used 50 combinations (*in vitro* vessels) with five true replicates (Halloran and Adelberg 2011) whereas a factorial of four factors at three levels per factor, with one mixture response factor at four levels, would result in 324 combinations, before any replication. The second experiment used 32 combinations *in vitro* (and 64 combinations in the greenhouse) with two true replicates, whereas a factorial of five factors at three levels per factor, the experiment duplicated for an additional qualitative factor at two levels, would result in 486 combinations, before replication. Sulfate was considered as a covariate in the experiment with P, Ca, and Mg as factors.

Design, analysis, and graphical visualizations were performed in JMP 10.0 (SAS Inst., Cary, NC). Model parameters were considered important (statistically significant) when a hypothesis test for H_o: model parameter=0 produced a P value<0.05. Parameters to be included in the final models were chosen using a forward stepwise procedure with the P value threshold to enter at 0.25 and threshold to leave at 0.10. Once the parameters to be included in the final model were obtained, a combination of (1) the model parameter P values, (2) the R^2 value, (3) the adjusted R^2 value (R^2_a), (4) the predictive R^2 value (R^2_p), (5) the overall F statistic and P value, and (6) the AIC value were used to assess the overall

model adequacy. We did not consider any one of the six criteria individually adequate to assess the model, and therefore used the combination. Interaction profilers were used to determine levels of the factors, in the presence of any interactions, which could be used to maximize the mean play responses. Model residuals were evaluated to assess the analysis assumptions and, fortunately, no issues were detected in the residuals that caused us to question the validity of the analysis results.

Results and Discussion

Two characteristics of micropropagation experiments allowed several experimental factors to be evaluated simultaneously
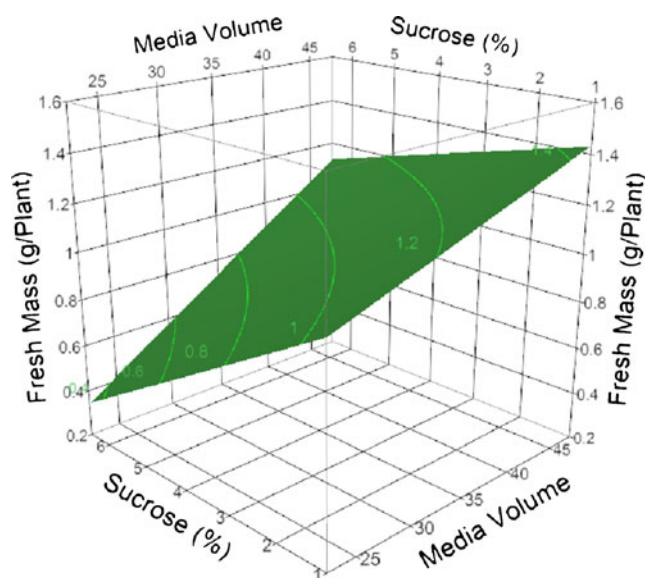

Figure 3. Response surface plot illustrates a subset of the plant size (gram of fresh mass/plant) model showing the interaction of media volume and sucrose concentration in experiment 1, with NH_4/K ratio set at 0, macronutrients at 98 mM, buds/vessel at three, and sucrose at 1.5%.

with a small subset of treatment factor combinations and limited replication. Based on a lack of evidence of higher order interactions in previous studies, a second-order response surface model was chosen to estimate, rank, and interpret the effects of the factors. This allowed the d-optimal criterion to be used to choose the small subset. The error control possible with stable clonal divisions in a reagent grade media in a controlled environment resulted in consistent plant responses from replication to replication and therefore a small number of replications were used.

Plant density, media volume, and macronutrient experiment. Multiplication rate. Several of the factors tested in this experiment affected the multiplication rate (Table 1). The most significant factor was buds/vessel with multiplication rate being higher at lower density (three buds/vessel). Media volume interacted with both sucrose concentration and macronutrient concentration. Multiplication rate was highest when media volume was around 40 mL and at the highest concentration of sucrose (6%) or macronutrients (100 mM with 50 mM $[NO_3^-]$=50 mM $([NH_4^+]+[K^+])$; (Fig. 1A, B). Plants in liquid culture were grown on an orbital shaker, and although increased media volume makes more nutrients and water available, a lack of aeration may eventually limit multiplication as media volumes increase. The model predicted the greatest multiplication rate (5.3×) with the lowest explant density (three buds/vessel), a high volume of medium (40 mL), and the most sucrose and macronutrients. In prior work with this clone of turmeric, the optimal multiplication rate was 3.4× in MS liquid with four plants per vessel in 33 ml medium with 4% sucrose (Adelberg 2010). The multiplication rate model in this experiment (Table 1) predicted 3.3 to 4.0× (95% confidence) for the set points four buds per vessel, 33 mL medium, 4% sucrose and MS macronutrients. There is good agreement between our model and prior published work. High-density cultures had reduced multiplication rates

during the micropropagation of other herbaceous perennials, *Alocasia*, *Colocasia*, *Hemerocallis*, and *Hosta*, in liquid medium (Adelberg and Toler 2004; Adelberg 2005; Adelberg et al. 2007).

New plants. Maximizing the output of new plants per vessel required starting with the maximum number (nine) of initial buds (Table 2). The most new plants came from a media volume of approximately 43 mL, and increasing media volume increased the optimal macronutrient ion concentration as well as the optimal sucrose concentration (Fig. 2A, B). We could expect 25.7 new plants/vessel in a high density culture (nine plants/vessel) with 43 mL medium, 6% sucrose and the most macronutrients (100 mM with 50 mM $[NO_3]$=50 mM $([NH_4^+]+[K^+])$. The multiplication rate limits production when the number of plants is limited, such as introduction of new plant cultivars, but production is often limited by the facility (culture room, autoclave, or laminar flow hood) and the number of new plants per vessel requires different optimization. However, higher-density cultures created a concern for the quality of plants produced.

Fresh weight per plant. Each of the five experimental factors significantly affected plant weight, and media volume and sucrose had a significant interactive effect on plant weight (Table 3). The largest plants (1.3 g/plant) came from vessels with the fewest initial buds (three), the greatest media volume (45 mL), and the lowest NH_4/K ratio (0, or all the macronutrients as KNO_3). The concentration of macronutrient ions showed a quadratic response, and the optima occurred very close to the maximum level tested 100 mM (with 50 mM $[NO_3^-]$=50 mM $([NH_4^+]+[K^+])$. The lowest sucrose concentration also increased the size of plants, likely due to increased uptake of water. In turmeric liquid culture, plant size (surface area), fresh mass, and water uptake were closely correlated to one another during a time course analysis of stage II

Table 4 Growth of turmeric plants in greenhouse after 14 day (fresh mass out/fresh mass in) as influenced by prior laboratory conditions during three 35-d cycles was modeled with terms that included: combined equivalence of three macronutrient ions (NH_4^+, K^+, NO_3^-), the proportion of NH_4^+/K^+, media volume, numbers of buds/vessel, and initial sucrose concentration. The final model had R^2=0.663, R^2_a=0.556, R^2_p=0.3874, overall model F ratio was 6.2021 (P<0.0001), and AICc was −57.049

| Model terms	Parameter estimate	P value[a]	Mean square
Macronutrients	−0.00327±0.0005	<0.0001	0.5622
NH_4/K ratio	−0.29177±0.0763	0.0004	0.1886
Media volume×buds/vessel	−0.00173±0.0006	0.0086	0.0984
Macronutrients×sucrose	0.00053±0.0002	0.0184	0.0778
Volume×volume	−0.00098±0.0004	0.0336	0.0623
Volume	0.003412±0.0018	0.064	0.0467
NH_4/K ratio×sucrose	−0.06453±0.0374	0.0922	0.0383
NH_4/K ratio×NH_4/K ratio	0.908163±0.5783	0.124	0.0318
Buds/vessel×sucrose	0.003854±0.0027	0.1661	0.0256
Macronutrients×macronutrients	2.95E−05±2.34E−05	0.2145	0.0205
Sucrose	−0.00949±0.0077	0.2234	0.0197
NH_4/K ratio×macronutrients	−0.00203±0.0024	0.3935	0.0096
Buds/vessel	−0.00478±0.0059	0.4187	0.0086

[a] P value of T test
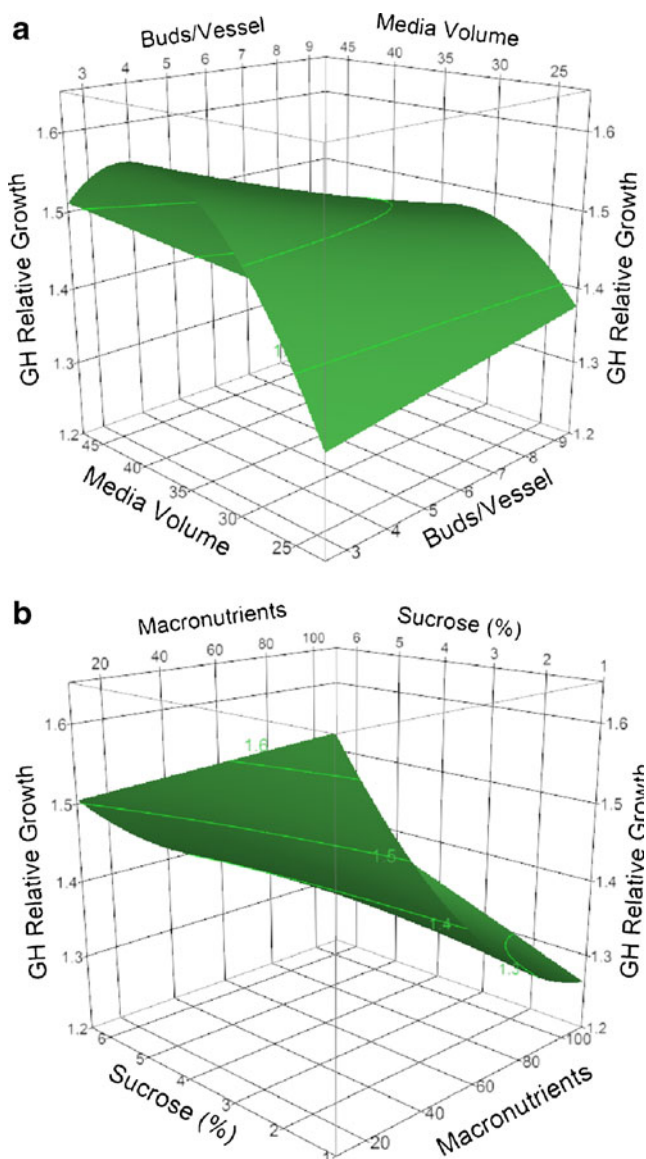
Figure 4. Response surface plot illustrates subsets of greenhouse growth (fresh mass on day 14/fresh mass on day 0) model in experiment 1: (*A*) buds/vessel×media volume with NH_4/K ratio set at 0, macronutrients at 20 mM, and sucrose at 1.5%; (*B*) macronutrients×sucrose (% m/v) with NH_4/K ratio set at 0, media volume at 38 mL, and buds/vessel at three.

multiplication (Adelberg 2010). Macronutrient ions and sucrose concentration both affected plant size as measured by
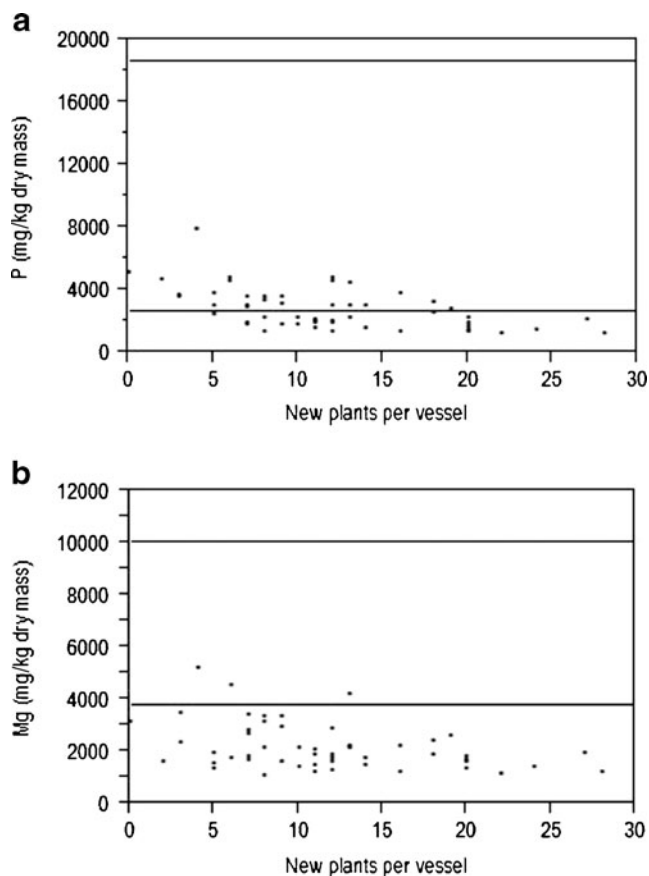

Figure 5. The elemental ratio of P (*A*) and Mg (*B*) in tissue dry mass was shown to be related to the numbers of new plants grown in each vessel in experiment 1. The *solid bars* show the reference standards for deficient and super-optimal nutrient concentrations in field grown turmeric established by Kumar et al. (2003).

fresh mass. The interaction between sucrose concentration and media volume showed that increased sucrose concentration was not a limitation in higher media volumes (Fig. 3). A larger amount of media (nutrients and water) allowed plants to grow in the more negative osmotic environment resulting from high sucrose concentrations. A maximized plant mass in a high-density culture of 0.78 g/plant (25.7 new plants/vessels) might still be preferred over the low-density 1.3 g per plant (13 new plants/vessel) if greenhouse performance was acceptable. If prior MS conditions for this clone (four buds, 4% sucrose,

Table 5 Maximum responses for multiplication rate, new plants/vessel, plant size, and greenhouse growth for turmeric following three 35-d cycles. Treatments associated levels of combined equivalence of three

macronutrient ions (NH_4^+, K^+, NO_3^-), the proportion of NH_4^+/K^+, media volume, numbers of buds/vessel, and initial sucrose concentration

Response	Maximum value	Media volume	Buds/vessel	Sucrose (%)	NH_4/K ratio	Macronutrients
Multiplication	5.3×	40	3	6	0.5	100
New plants	25.7	43	9	6	0.5	100
FW/plant	1.3 g	45	3	1.5	0	98
Greenhouse growth	1.6×	39	3	1.5	0	20

33 mL medium; Adelberg 2010) were combined with high plant densities, the plant size model (Table 3) predicted plant size would be reduced to 0.19 to 0.52 g/plant (95% confidence).

Greenhouse growth ratio. More than 95% of the 952 plants transferred from *in vitro* culture successfully acclimatized to greenhouse conditions. The ability to grow during acclimatization was affected by the prior *in vitro* conditions. The macronutrient concentration was the most significant factor (Table 4), with greenhouse growth increasing with lowered concentration. NH_4/K ratio was also a significant main effect, with the most growth occurring with 0 NH_4^+ (all macronutrient cations as K^+). With ammonium and macronutrient concentrations low, at high plant density there were minimal effects of media volume and all plants increased by about 40% (Fig. 4A). The most growth came from the fewest buds/vessel, the least macronutrients, no ammonium, and 39 mL medium, with plant mass increasing 58% during 14 d of greenhouse acclimatization.

Attempting to find an optimal medium by selecting the best treatments had some apparent conflicts (Table 5). A medium volume around 40 mL was a reasonable number (25 vessels/l) for maximal multiplication and growth in greenhouse. The high sucrose, NH_4NO_3 multiplication medium would not be a good medium for plant size or greenhouse growth. Reducing the number of plants to achieve larger-sized plants would not be an economic solution since each plant has a base value, with little differential for size. Harmonizing these models for sucrose or macronutrients did not allow consensus.

Spent medium analysis. Reducing the amount of buds in a vessel to increase size, growth rate, or multiplication rate suggested that something was limiting growth that was not in the model. When analyzing nutrient content in spent medium

Table 6 Multiplication rate of turmeric following three 35-d cycles was estimated by a model with terms that included: P, Ca, Mg, KNO_3, and plant density. The final model had $R^2 = 0.553$, $R^2_a = 0.441$, $R^2_p = 0.2866$, overall model F ratio was 5.800 ($P < 0.0001$), and AICc was 135.630

Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	-0.1029 ± 0.0257	0.0002	5.1914
P mM	0.0947 ± 0.0328	0.0055	2.6939
Ca mM $\times$ KNO_3 mM	0.0038 ± 0.0014	0.0120	2.1757
P mM $\times$ P mM	-0.0805 ± 0.0319	0.0145	2.0558
Mg mM $\times$ buds/vessel	0.0447 ± 0.0185	0.0188	1.8893
Ca mM	-0.0529 ± 0.0251	0.0396	1.4335
P mM $\times$ buds/vessel	0.0226 ± 0.0111	0.0459	1.3462
Mg mM $\times$ Mg mM	0.1349 ± 0.0901	0.1399	0.7237
Ca mM $\times$ buds/vessel	0.0118 ± 0.0095	0.2194	0.4980
KNO_3 mM	0.0024 ± 0.0039	0.5295	0.1293
Mg mM	-0.0068 ± 0.0508	0.8941	0.0058

[a] P value of T test

following the 35-d culture period, phosphorus and magnesium were most depleted (compared to the elemental standards for field-grown turmeric established by Kumar et al. 2003). For instance, in field-grown turmeric, P was optimal in the range of 2,500 to 15,600 ppm. Plants from the majority of vessels in this experiment (31 of 55) fell below the standards of P adequacy established for turmeric (Fig. 5A). There was an inverse relationship between the number of new plants produced and the P content of those plants ($r = -0.5286$; $P < 0.0001$) and every vessel with 20 or more new plants had sub-optimal concentrations of P in the plant tissue. Similarly, Mg was inadequate in 51 of 55 vessels and an inverse relationship existed between the numbers of new plants produced and the Mg content of those
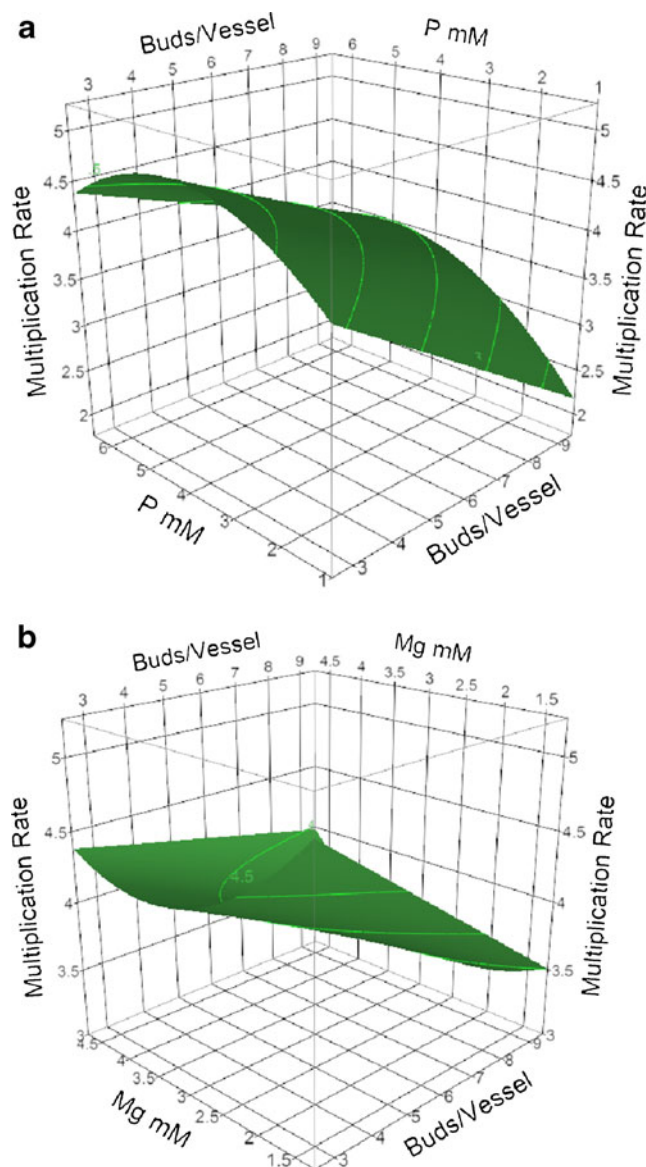
Figure 6. Response surface plot illustrates subsets of the multiplication model in experiment 2: (*A*) effect of buds/vessel × P mM with calcium set at 3 mM, magnesium at 1.5 mM, and KNO_3 at 20 mM; (*B*) buds/vessel × Mg mM with phosphorus set at 4.1 mM, calcium at 3 mM, and KNO_3 at 20 mM.

plants ($r = -0.4231$; $P = 0.002$; Fig. 5B). Spent medium analysis following micropropagation in liquid showed that the P concentration in MS was inadequate for turmeric (Adelberg 2010), and both P and Mg concentrations were inadequate for *Hemerocallis* (Adelberg et al. 2010). High-density cultures were more acutely affected by inadequate nutrients than low-density cultures (Adelberg et al. 2010).

Mesonutrient, macronutrient, and plant density experiment. A second multifactor experiment was conducted where P was increased as much as five times the original MS concentration (1.25 to 6.25 mM) and Mg was increased as much as three times the original MS concentration (1.5 to 4.5 mM). Calcium was also varied since P and Mg may antagonize Ca availability. Ammonium was supplied at a low concentration (5 mM) and the other macronutrients were supplied as KNO_3 (20 to 60 mM). There were three to nine buds/vessel. Media volume was constant at 40 mL and sucrose was constant at 5%.

In this experiment, P and Mg in tissue dry mass was increased by raising the concentration of these elements in the media ($P > 0.0001$ for both nutrients). Increasing P from 1.25 mM (original MS concentration) to 3.75 or 6.25 mM brought P in tissue dry mass into the adequacy range for plants in 40 of 40 vessels. Mg was improved to a lesser degree. At MS and 2× MS concentrations (1.5 and 3.0 mM), Mg was at or near deficiency in 32 of 34 vessels. At 3× MS concentrations (4.5 mM) Mg was adequate in 10 of 26 vessels. The adequacy and deficiency levels determined by Kumar et al. (2003) were developed in field-grown turmeric plants and only suggestive of *in vitro* performance.

Multiplication rate. Buds/vessel was the most significant factor in the model (Table 6), with multiplication rate increasing

with lower bud density. Phosphorus was significant as a main and quadratic term with the maximum multiplication rate occurring at 4.11 mM. Buds per vessel showed interactions with phosphorus and magnesium. The reduction in multiplication rate at high bud densities was less severe at high P concentrations (Fig. 6A). The MS level of 1.5 mM Mg was optimal when buds/vessel was low, but multiplication rate decreased sharply as buds/vessel increased (Fig. 6B). At 4.5 mM Mg, buds/vessel increased with little effect on multiplication rate. The maximum multiplication rate of 4.7× (4.1× to 5.4×, 95% confidence) was not any higher than the maximal amount of 5.3× in the prior

Table 7 New plants of turmeric per vessel following three 35-d cycles was estimated by a model with terms that included: P, Ca, Mg, KNO_3, and plant density. The final model had $R^2 = 0.851$, $R^2_a = 0.818$, $R^2_p = 0.7702$, overall model F ratio was 26.097 ($P < 0.0001$), and AICc was 344.837

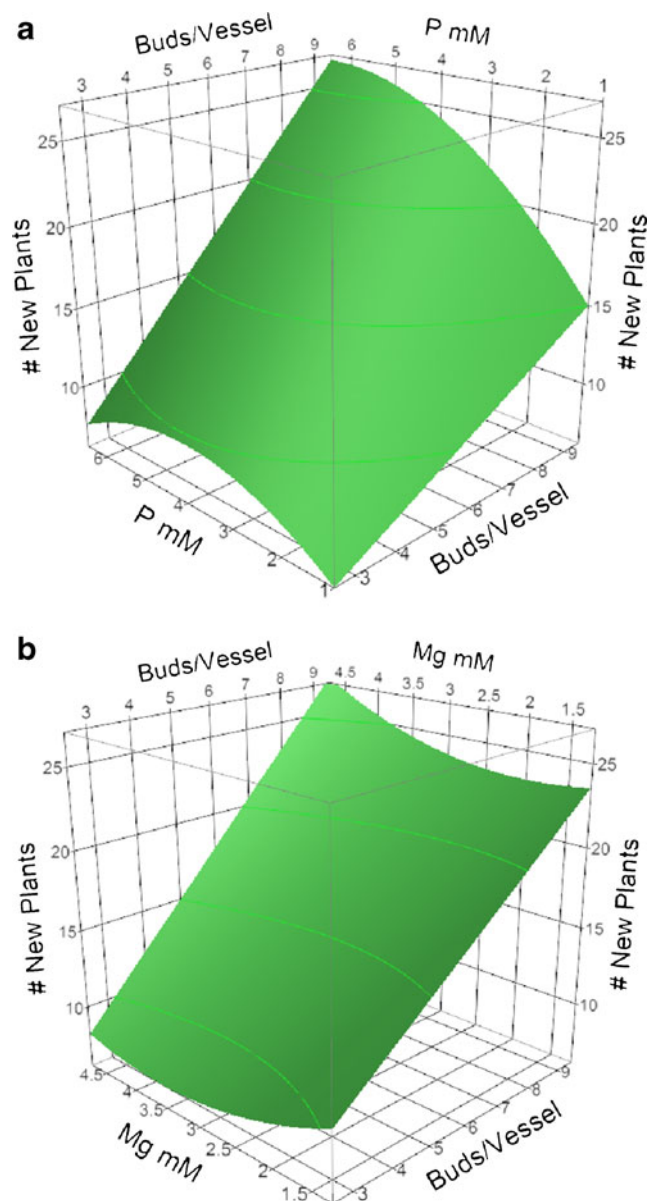| Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	1.7250 ± 0.1176	<0.0001	1464.3134
P mM	0.8130 ± 0.1502	<0.0001	199.2139
P mM × buds/vessel	0.2668 ± 0.0513	<0.0001	184.0661
Mg mM × buds/vessel	0.2432 ± 0.0857	0.0063	54.7818
Ca mM × KNO_3 mM	0.0184 ± 0.0065	0.0065	54.3704
P mM × P mM	-0.3875 ± 0.1451	0.0100	48.4818
Ca mM	-0.2705 ± 0.1166	0.0241	36.5935
Mg mM × Mg mM	0.5512 ± 0.4096	0.1839	12.3163
P mM × KNO_3 mM	0.0101 ± 0.0077	0.1971	11.5951
Ca mM × Ca mM	-0.1274 ± 0.1014	0.2143	10.7370
Mg mM	0.2831 ± 0.2321	0.2279	10.1146
KNO_3 mM	0.0138 ± 0.0176	0.4381	4.1499

[a] P value of T test

Figure 7. Response surface plot illustrates subsets of the model for number of new plants/vessel) in experiment 2: (A) buds/vessel × P mM with calcium set at 6.7 mM, magnesium at 4.5 mM, and KNO_3 at 60 mM; (B) buds/vessel × Mg mM with phosphorus set at 6.25 mM, calcium at 6.7 mM, and KNO_3 at 60 mM.

experiment (that did not adjust mesonutrient concentrations for high bud density). Increasing buds per vessel caused less of a decline in multiplication rate with elevated P and Mg.

New plants. Buds/vessel was the most significant factor affecting the number of new plants per vessel (Table 7), with the number of new plants increasing at higher density. Phosphorus was again significant as a main effect and as a negative quadratic effect. It may not be advantageous to increase P past 6.25 mM, the highest concentration tested in this experiment. Buds/vessel also showed an interaction with phosphorus. High bud density drove an increased need for P to produce more new plants, while P had a negligible effect at three buds/vessel (Fig. 7A). Here, the greater demand for P concentration coincided with the demand created by initial buds in a fixed volume, and so the optimal P concentration for new plants (6.25 mM) was higher than the optimal value for P concentration for multiplication (4.11 mM) at low density. The interaction of buds/vessel and Mg (Fig. 7B) showed that increasing Mg had little effect at three buds/vessel, but there was a slight positive effect at nine buds/vessel. Again, the demand for Mg coincided with increased buds in a fixed volume. Lowering Ca concentration allowed for more new plants to be produced, and Ca interacted with KNO$_3$. In both the multiplication rate model and new plants model, when KNO$_3$ was low, increasing Ca had a negative effect. However, when KNO$_3$ was high, there was no calcium effect (data not shown). The maximum number of new plants/vessel, 25.4, was nearly identical to the first experiment.

Fresh weight per plant. Fresh weight/plant increased with reduced density, and buds/vessel was the most significant main effect (Table 8). Potassium nitrate and P were both shown to have main effects with positive quadratic terms, so

increasing either produced a higher fresh weight per plant. Optimal plant size was at the highest levels tested, so extending the ranges tested may be beneficial. Potassium nitrate interacted with buds/vessel, as a high concentration (60 mM) produced a greater impact on fresh weight than a low concentration (20 mM) as buds/vessel was decreased. Magnesium and Ca had quadratic effects with negative terms, so their optimal concentrations fell within the ranges tested. Interactions were not significant and contour surfaces show the quadratic responses to Mg, Ca, and P concentrations (Fig. 8A, B). The largest plants that grew to 1.7 g/plant (1.5 to 1.9 g, 95% confidence) came from the three buds/vessel

Table 8 Plant size of turmeric following three 35-d cycles was estimated by a model with terms that included: P, Ca, Mg, KNO$_3$, and plant density. The final model had $R^2 = 0.708$, $R^2_a = 0.657$, $R^2_p = 0.5915$, overall model F ratio was 13.811 ($P < 0.0001$), and AICc was -11.244

| Model terms	Parameter estimate	P value[a]	Mean square
Buds/vessel	-0.0631 ± 0.0088	<0.0001	1.9591
KNO$_3$ mM	0.0066 ± 0.0014	<0.0001	0.9022
P mM	0.0509 ± 0.0113	<0.0001	0.7783
KNO$_3$ mM × KNO$_3$ mM	0.0005 ± 0.0002	0.0029	0.3708
Mg mM × Mg mM	-0.0935 ± 0.0309	0.0038	0.3496
P mM × P mM	0.0295 ± 0.0108	0.0084	0.2855
Ca mM × Ca mM	-0.0176 ± 0.0076	0.0234	0.2077
KNO$_3$ mM × buds/vessel	-0.0011 ± 0.0005	0.0240	0.2057
Ca mM	-0.0052 ± 0.0087	0.5556	0.0135
Mg mM	0.0049 ± 0.0174	0.7808	0.0030

[a] P value of T test
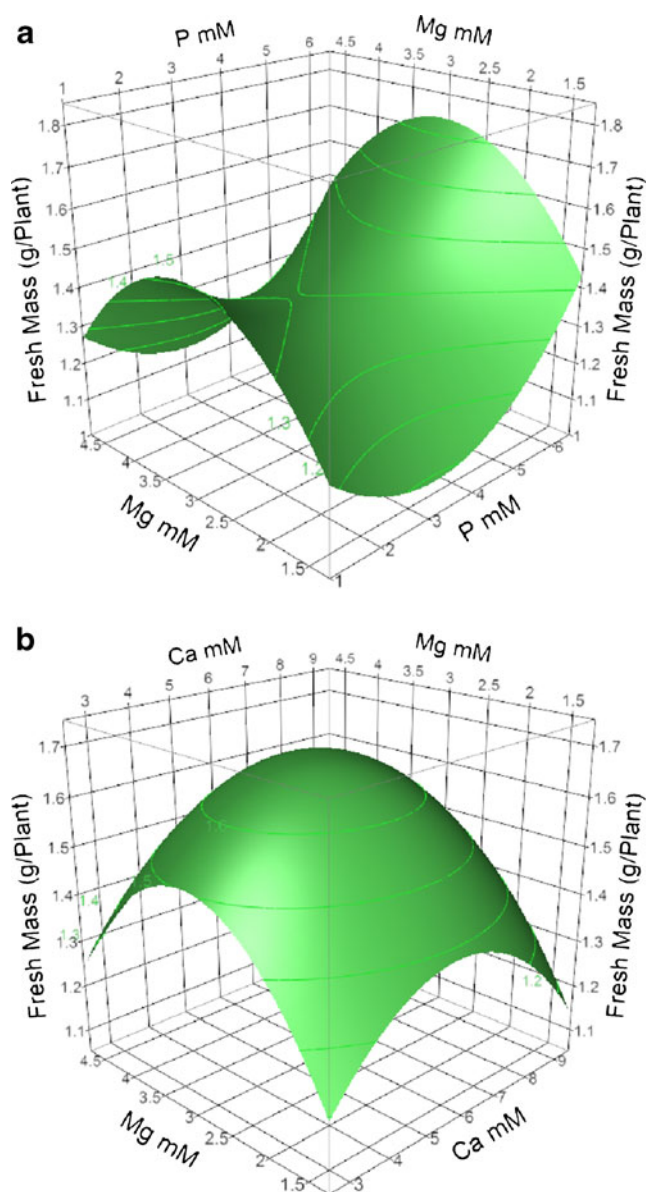
Figure 8. Response surface plot illustrates subsets of the plant size (gram of fresh mass/plant) model in experiment: (A) P mM and Mg mM with calcium set at 5.76 mM, KNO$_3$ at 60 mM, and buds/vessel at three: (B) Ca mM and Mg mM with phosphorus set at 6.25 mM, KNO$_3$ at 60 mM, and buds/vessel at three.

Table 9 Growth of turmeric plants in greenhouse after 14 day (fresh mass out/fresh mass in) was influenced by prior laboratory conditions during three 35-d cycles as estimated by a model with terms that included: P, Ca, Mg, KNO_3, and plant density. The final model (developed with a forward stepwise procedure) had $R^2=0.332$, $R^2_a=0.212$, $R^2_p=-0.0656$, overall model F ratio was 2.778 ($P<0.0074$) and AICc was 135.325

Model terms	Parameter estimate	P value[a]	Mean square
P mM×P mM	−0.1033±0.0320	0.0021	3.5188
KNO_3 mM×KNO_3 mM	−0.0012±0.0005	0.0184	1.9972
P mM	−0.0841±0.0348	0.0189	1.9780
Mg mM×Mg mM	0.1620±0.0922	0.0844	1.0452
Buds/Vessel	0.0412±0.0266	0.1275	0.8102
P mM×buds/vessel	−0.0168±0.0115	0.1508	0.7181
Greenhouse fertilizer [no]	−0.0963±0.0712	0.1815	0.6197
Greenhouse fertilizer [no]×Mg mM	−0.0690±0.0514	0.1852	0.6090
KNO_3 mM	−0.0038±0.0040	0.3484	0.3027
Mg mM	0.0046±0.0516	0.9287	0.0027

[a] P value of T test

treatment, and this was greater than the maximal value for the prior experiment (1.3 g/plant). More importantly, the high-density/high-yield cultures initiated with nine buds/vessel, with an optimal nutrient formulation of P (6.25 mM), Ca (5.8 mM), Mg (3.1 mM), and KNO_3 (60 mM), yielded 1.1 g/plant (0.9 to 1.3 g 95% confidence) that was nearly equal to the maximum value from low-density cultures (1.3 g/plant) in the prior experiment. High-density cultures now yielded almost twice as many large plants in a fixed volume of medium than low-density cultures in the prior experiment, where density and macronutrients were considered without adjusting for mesonutrients.

Greenhouse growth ratio. Upon transfer to the greenhouse, more than 95% of the 769 plants survived. The advantage of proper nutrition was observed as enhanced growth on the mist bench during the first 14 d of growth. Phosphorus and KNO_3 had negative quadratic terms and optimal concentrations were within the ranges tested, at 3.32 and 37 mM, respectively, or approximately 3× and 2× of the original MS formulation (Table 9, Fig. 9). The greatest greenhouse growth, 2.4× to 3.6× (95% confidence), was in treatments with starter fertilizer in the greenhouse soilless medium, but that may not have been significantly different from greenhouse media without fertilizer (2× to 3.2×, 95% confidence). The uptake of nutrients by plants in the lab is more important than nutrients supplied by the soilless mix during the first 14 d on the mist bench. More new plants/vessel did not have a negative impact the growth of plants in the greenhouse. Better mineral nutrition *in vitro* allowed more plants to be grown to larger sizes in a defined volume of laboratory medium, and upon subsequent *ex vitro* transfer had the most vigorous growth in the greenhouse.

However, the greenhouse growth model had poor fit compared to laboratory experiments previously presented. The environment on the greenhouse mist bench was less uniform than what is found in a vessel in a laboratory. Better resolution of greenhouse responses may require larger sample sizes, longer growth periods, or more precise measurements. For

instance, the short duration of growth (14 d) was chosen, because removing soil from the root system to measure root mass was likely source of error, and we hoped a small root at 14 d would help to eliminate this error. Alternative methods to standardize the greenhouse environment and measure greenhouse growth may be important. Notwithstanding, *in vitro* mineral nutrition improved subsequent greenhouse growth and had a greater impact than fertilizer applied in the greenhouse medium.

We have observed that interactions between nutrients are significant factors in the formulation of growth media. It is also apparent that process variables in micropropagation, like bud density or media volume, will also impact the optimal concentration of nutrients in a media formulation. Optimal media formulae will change depending on
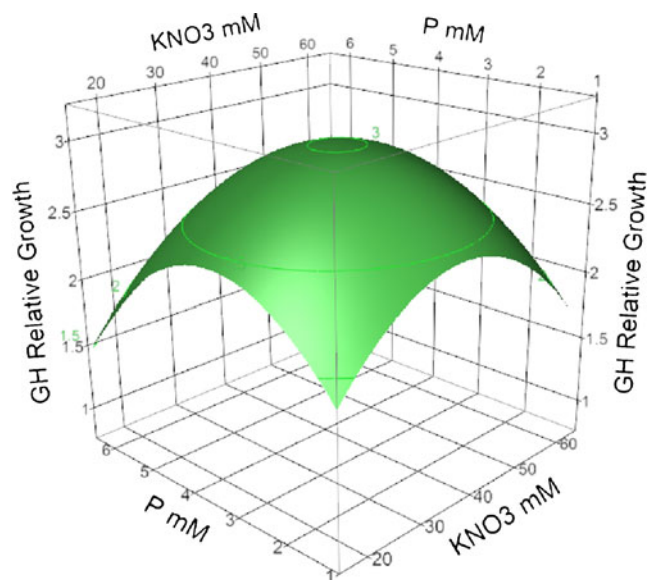

Figure 9. Response surface plot greenhouse growth (fresh mass on day 14/fresh mass on day 0) model in experiment 2 showing P mM×KNO_3 mM with greenhouse starter fertilizer present, calcium set a 5.8 mM, magnesium 4.5 mM, and buds/vessel at nine.

Table 10 Maximum responses for multiplication rate, new plants/vessel, plant size, and greenhouse growth and associated levels of P, Ca, Mg, KNO$_3$, and plant density

Response	Maximum value	Buds/vessel	Phosphorus (mM)	Calcium (mM)	Magnesium (mM)	KNO$_3$ (mM)	Greenhouse fertilizer
Multiplication	4.8×	3	4.11	3	1.5	20	N/A
New plants	25.4	9	6.25	6.0	4.5	60	N/A
FW/plant	1.7 g	3	6.25	5.8	3.1	60	N/A
Greenhouse growth	3×	9	3.32	3–6	4.5	37.5	Yes

whether low-density rapid multiplication, high-density maximum yield, large plant size, or rapid growth of plants in greenhouse is most important (Table 10). Consensus media may be calculated for stages II and III at high density. For instance, a stage II medium with nine buds/vessel, 5.5 mM P, 3 mM Ca, 4.5 mM Mg, and 38 mM KNO$_3$ would yield 25 new plants and a multiplication ratio of 4×. A stage III medium to maximize greenhouse growth could have less P (3.3 mM) than the stage II medium (with the other nutrients remaining about the same) and plants would more than double in mass in the greenhouse.

The concentrations of P, Ca, Mg, and KNO$_3$ in these media were most often higher than MS (1.25 mM P, 3 mM Ca, 1.5 mM Mg, 20 mM K, and 40 mM NO$_3$), and all were higher than ½ MS (0.6 mM P, 1.5 mM Ca, 0.7 mM Mg, 10 mM K, and 20 mM NO$_3$). Ammonium in MS is about 20 mM (10 mM in ½ MS), and was 5 mM in all the media formulated in the second experiment (to choose mesonutrient, macronutrient and plant density), so we suggest that reduced ammonium may be the benefit others realize from choosing ½ MS stage III medium. Phosphorus and Mg in MS medium were inadequate for turmeric in any situation, and a diluted MS medium is far from optimal as a stage III medium for turmeric. Shoot quality was improved by approximately doubling the P, Ca, and Mg in a comprehensive screening of mineral nutrition in media for pear shoot cultures (Reed et al 2013; Wada et al 2013). Lowering NH$_4$NO$_3$ to ½ MS concentration increased shoot multiplication in most cultures, and KNO$_3$ had little effect on shoot numbers.

Conclusions

MS medium is known for its high concentration of inorganic nitrogen (60 mM), with about a 2:1 ratio of NO$_3^-$ to NH$_4^+$. Altering inorganic nitrogen is the most frequently reported modification of MS formulae (George and De Klerk 2009). In our current work, inorganic nitrogen sources at MS concentrations allowed good rates of multiplication. However, larger plants with better growth in the greenhouse came from media where NH$_4^+$ was eliminated. In a meta-analysis of adjustments to MS medium to improve shoot cultures, two plant species benefitted from lowering the ratio of NO$_3^-$/NH$_4^+$ to approximately 1:1, and

14 plant species benefitted by raising NO$_3^-$/NH$_4^+$ to 2:1 or greater (George and De Klerk 2009). Turmeric appears to be in the latter group.

A unique aspect of our current work shows how variation in the tissue culture process, namely, the number of buds and medium volume in a vessel, will affect the optimal concentration of nutrients required. For example, an optimal concentration of macronutrients could not be specified without considering the process variables: media volume and the amount of tissue used to initiate a vessel (Tables 5 and 10). It is intuitive that high-density cultures with the greatest input (nine buds/vessel) require the greatest amounts of sucrose (6%) and nutrients (media volume) for optimal multiplication. Reducing the number of initial plants increased the multiplication rate and increasing the amount of macronutrients did not alter the multiplication response. Therefore, nutrient elements in media not included as independent variables in experiment may increase growth. In the subsequent experiment, additional P and Mg produced larger plants at higher densities and these plants grew rapidly in the greenhouse, but the multiplication rate was not improved.

Prior work with a full, five factor design that included plant density and nutrition during liquid-based micropropagation showed third-, fourth-, and fifth-order interactions had much less significance than first- and second-order effects (Adelberg et al. 2010). The response surface models (limited to second-order interactions) used in our current two experiments had good predictive value for the numbers of new plants and the size of the new plants. The models for multiplication rate in both experiments, and greenhouse growth in the mesonutrient, macronutrient and plant density experiment, had lower predictive value. This experimental design may need more precise greenhouse culture methods so the difference in responses between treatment factor levels, and therefore, the reliability of the model, would be improved.

Media formulae do not offer any "one size fits all" solutions to resolve problems of efficient micropropagation practice. Synergistic and antagonistic responses among factors would have been missed in the prior generation of media formulae developed by OFAT methods based on salt concentrations. Murashige and Skoog (1962) speculated that water was the limitation to growth in their new media. Nutrients are not readily available if the availability of water is restricted.

Liquid medium makes water and solutes more available than agar based medium resulting in greater *in vitro* growth in many systems (Adelberg and Fari 2010). Modern statistical optimization designs have the potential to greatly increase efficiency if process, nutrients, and the different stages of development are considered using multifactor approaches. Although it is likely that the media suggested in Table 10 should outperform MS with other herbaceous perennials, it is our opinion that conducting multifactor medium optimization for each species would provide better results. Economically important crops certainly warrant this type of analysis.

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