Plant Cell Tiss Organ Cult (2016) 126:33–42
DOI 10.1007/s11240-016-0974-9

ORIGINAL ARTICLE

Optimizing in vitro mineral nutrition and plant density increases greenhouse growth of *Curcuma longa* L. during acclimatization

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Received: 19 November 2015 / Accepted: 9 March 2016 / Published online: 16 March 2016

Abstract The growth of plantlets during hardening is affected by the tissue culture media in which they were grown. A multi-factor approach to optimizing nutrient ions in media for subsequent *ex vitro* growth has not been studied. A response surface method was designed to test in vitro plant density (50–250 buds/L), PO_4^{3-} (6.25–10.25 mM), Ca^{2+} (2–10 mM), and KNO_3 (18–100 mM) in low NH_4^+ on medium as it affected subsequent growth in a 21 days acclimatization period with *Curcuma longa* genotypes (L35-1 and L22-5). The treatment media were compared with the MS medium. Plants grown on treatment media had higher relative fresh biomass (3.3 ± 0.5 fold from high-density with 42 mM KNO_3; 3.7 ± 0.5 fold from low-density with 18 mM KNO_3) than MS plants (2.6 ± 0.5 fold) regardless of plant density. Leaf area index from the treatments (67.0 ± 16.4 cm^2/bud from high-density with 70.4 mM KNO_3; 78.4 ± 16.4 cm^2/bud from low-density with 50.7 mM KNO_3) was higher than MS plants (22.0 ± 8 cm^2/bud from high-density and 36.0 ± 8 cm^2/bud from low-density). Shoots from treatment plants receiving 64 mM KNO_3, elongated to 8.6 ± 0.9 cm from high-density and 10.9 ± 0.9 cm from low-density. Genotype L22-5 elongated to 6.0 ± 0.6 cm regardless of density, and L35-1 elongated to 6.0 ± 0.6 cm from low-density and to 4.0 ± 0.6 cm from high-density. Raising phosphate 5× to 10× the MS concentration in the pre-hardening media did not influence *ex vitro* responses. Plants grown on modified low ammonium media can grow faster and larger during acclimatization with increased KNO_3, especially from high-density culture that would be preferred in commercial applications.

Keywords Murashige and Skoog medium · Mineral nutrition · Turmeric · Acclimatization · Response surface design · D-optimal criteria

Introduction

The benefits of micropropagation can be valued with successful plant growth in the greenhouse (Hazarika 2003). The regulation of environmental conditions including light intensity, CO_2, sucrose concentration, and nutrients supplied during in vitro culturing, may improve the plant growth of certain species when they are transferred to *ex vitro* conditions (Adelberg et al. 2015; Barrales-López et al. 2015). Since growth of plant organs has different nutrient requirements (Ramage and Williams 2002; El-Hawaz et al. 2015a), use of MS medium (Murashige and Skoog 1962) for *ex vitro* subsequent growth may not be suitable (Adelberg et al. 2007, 2010). In addition, different plant genotypes might require specific in vitro nutrition (Williams 1993; Leifert et al. 1995; Gahan 2008). Multi-factor experiments using response surface methods (RSM) show the optimal mineral concentrations for *ex vitro* growth were different than in vitro growth for turmeric (Adelberg et al. 2013; El-Hawaz et al. 2015b). The concentrations of PO_4^{3-} and KNO_3 in pre-hardening in vitro media have a greater impact than applying fertilizer on transplants grown in a soilless mix during a 2 week acclimatization in a greenhouse, however the random error

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due to variations in irrigation, wind and light distribution on the mist bed resulted in low accuracy models (Adelberg et al. 2013). Since most of the nutrients required for subsequent growth during acclimatization came from the medium used during in vitro pre-hardening, diluting the medium may limit the subsequent *ex vitro* growth Rooting and plant quality for acclimatization may be improved by lowering NH_4^+ in MS medium (Economou 2013). In turmeric, NH_4^+ free medium resulted in the best greenhouse growth during acclimatization, when NO_3^-, PO_4^{3-}, Ca^{2+} and Mg^{2+} were present at fairly high concentrations compared with MS (Adelberg et al. 2013).

The MS medium has sixteen elements, along with organic compounds and plant growth regulators (Murashige and Skoog 1962). Choosing which elements to vary in an experiment should be a deliberate strategy. Niedz and Evens (2007) clustered mineral salts in four groups: macronutrients (NH_4NO_3 and KNO_3), mesonutrients (KH_2PO_4, $MgSO_4$, and $CaCl_2$), micronutrients ($CuSO_4$, KI, $CoCl_2$, H_3BO_3, and Na_2MoO_4) and chelated iron, and then screened the factors in high and low concentrations relative to MS using RSM. Using this design, MS mineral salts were screened for improved in vitro shoot and leaf quality of *Rubus idaeus* L. (Poothong and Reed 2014), *Corylus avellana* L. (Hand et al. 2014), and *Pyrus* cultivars (Reed et al. 2013a, b) during stage II of the micropropagation process. Other RSM experiments sufficiently identified the interactive effects of mesonutrient combinations on in vitro responses of *Pyrus* cultivars (Wada et al. 2013, 2015) and *Curcuma longa* (Adelberg et al. 2013; El-Hawaz et al. 2015a). Testing salts as factors caused ion-confounding results and the specific concentration effect of each element could not be specifically quantified (Niedz and Evens 2006, 2008). By balancing the charge of ions, Niedz and Evens (2008) determined a synergistic interaction of NH_4^+ and K^+ that affected the in vitro growth of *Citrus sinensis* (L.) Osbeck. Low NO_3^- and low NH_4^+:K^+ increased the growth of *Brugmansia* × *candida* Pers. (Niedz et al. 2012) and equimolar of NH_4^+:K^+ enhanced the quality of *Gerbera hybrid* (Niedz et al. 2014). In a similar experimental design, the subsequent greenhouse growth of *C. longa* was maximized when NO_3^- was the only source of N in the in vitro pre-hardening medium (Adelberg et al. 2013).

Doubling the standard MS concentration of in vitro PO_4^{3-} from 1.25 to 2.5 mM increased the growth of *Asparagus officinalis* L. shoot apex culture (Murashige et al. 1972). With *C. longa*, phosphorus was the first mineral to be depleted from the 2.5 mM PO_4^{3-} medium, Murashige et al. (1972), during the 21 days micropropagation period (Adelberg 2010). The in vitro growth of *Musa* spp. was enhanced with raising PO_4^{3-} from 1.25 to 2 mM in solid MS medium (Amiri 2008). *Solanum tuberosum* L. in vitro growth and shoot length increased with 2.5 and 6.25 mM PO_4^{3-} in MS medium (Sarkar et al. 2004). Increasing the concentration of NO_3^- beyond the standard MS concentration improved the in vitro growth of *Eucalupyus* (Ivanova and Van Staden 2009), *Prunus domestica* L. (Nowack et al. 2007), and *Eleutherococcus koreanum* Nakai. (Lee and Paek 2011).

Liquid culture has inherent advantages for studying mineral nutrition when compared to semi-solid (agar-type) medium (De Klerk and Brugge 2011) including the elimination of gradients within the medium and greater water availability to the plant. High plant density culture is desirable for commercial utility (Leifert et al. 1991). In liquid cultures, increased density exhausted PO_4^{3-}, K^+, and N in medium and reduced N and PO_4^{3-} concentrations in *Delphinium* (Lumsden et al. 1990), *Hemerocallis*, and *Iris* tissue (Leifert et al. 1991). Spent medium analysis was used to show increasing in vitro plant density decreased PO_4^{3-} and Mg^{2+} in *Hemerocallis* tissue to levels that were below standards of adequacy for subsequent nursery growth (Adelberg et al. 2010). By including plant density as a factor in the RSM, increased plant density was shown to increase the need for more minerals in order for *C. longa* to grow faster when acclimatized in the greenhouse (Adelberg et al. 2013; El-Hawaz et al. 2015b).

Turmeric, *C. longa,* is a sterile perennial rhizomatous plant, and an economically important species within the Zingiberaceae family. Turmeric powder has a market value as a condiment food color and flavoring agent (Madan 2007) and is used in folk medicines of Asia and Middle East (Afifi and Abu-Irmaileh 2000; Latif et al. 2014). Turmeric was chosen as a model to develop media for herbaceous monocots used in phytochemical production or perennial ornamental plant micropropagation (Adelberg 2010).

The current research aims were (1) to observe the effects of low NH_4^+ medium containing various mineral concentrations on the subsequent growth after 21 days acclimatization for two genotypes of *C. longa* (2) to identify optimal combinations of in vitro minerals and plant density for *ex vitro* relative gain in fresh biomass, leaf area index, and the shoot length, and (3) to compare these results to what is observed from liquid MS medium.

Materials and methods

Plant material

Curcuma longa genotypes L35-1 and L22-5 rhizomes were sterilized and initiated in stage I as described by Adelberg and Cousins (2006). The genotypes were supplied by the University of Arizona Southwest Center for Natural Products Research and Commercialization (UA Herbarium #375,742, ARIZ). Turmeric buds (0.5–1 cm) were

micropropagated as stage II for 10 years by repeated subculture in 33 mL of MS medium including 100 mg myo-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine HCl, 6 % w/v sucrose and 1 μM benzyladenine (BA) in a cylindrical glass jar (180 mL) with a Magenta B-cap closure (Magenta Crop, Chicago, IL, USA) on an orbital shaker (100 rpm) at 23 ± 2 °C under cool white fluorescent lights with the intensity of 25–30 μmol m^2 s^{-1} photosynthetically active radiant (PAR) for 16 h per day. Then each five buds were subcultured in 40 mL of 2 × SH medium (Schenk and Hilderbrandt 1972) supplied with 5 % w/v sucrose and 3 μM BA for three cycles 35 days each for adapting plantlets to the high mineral concentrations in treatment media.

Response surface methods for in vitro propagation

Turmeric buds were transferred into 40 mL of low ammonium (5 mM NH_4^+) MS medium with 5 % sucrose, 3 μM BA (with vitamins and inositol, as above) and with twenty-five combinations of PO_4^{3-} (6.25, 8.25, 10.25 mM), KNO_3 (18, 59, 100 mM), Ca^{2+} (2, 6, 10 mM) in different densities (50, 150, 250 buds/L) as shown in Table 1. To maintain the charge balance in media mixtures, SO_4^{2-} ion was used as a counter ion and considered as a covariant factor (Adelberg et al. 2013; El-Hawaz et al. 2015a). Media pH range was measured but not controlled (5.26–5.98), since adjusting pH by NaOH would introduce Na^+ as a cofounding counter ion. At the same time, the genotypes were also propagated at the three densities in 40 mL of liquid basal MS medium (1962) with 5 % w/v sucrose and 3 μM BA as a control. All media included vitamins and inositol (as described above). Trimmed buds were subcultured for four 35 days cycles for adapting plantlets to treatment conditions and were grown on orbital shakers with lighting and temperature, as described above

Media analysis

At the end of the fourth cycle, the concentration of PO_4^{3-}, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Fe^{3+}, Mn^{2+}, B^{3+}, Zn^{2+}, and Cu^{2+} in the remaining media were determined by Thermo Jarrell Ash Model 61E Inductively Coupled Plasma (ICP; Analytical West, Inc., Corona, CA, USA). A Flow Injection Analyzer (FIAlab-2500; FIAlab instruments, Inc., Bellevue, WA, USA) determined N in NO_3^-. The Agricultural Service Laboratory at Clemson University (Clemson, SC, USA) provided the mineral analysis.

Acclimatization

Following the fourth cycle, the rooted in vitro plantlets from each vessel were washed with tap water and assigned to planting trays (601, six sections L × W × H, 16 × 12 × 5.5 cm polyethylene insert, fitting a webbed 1020 polyethylene tray, Landmark Plastics, Akron, OH, USA) based on the initial plant density as follows: the products of low plant density (50 buds/L) were transferred to one section, the products of moderate plant density (150 buds/L) were transferred to 3 sections, and the products of high plant density (250 buds/L) were transferred to 5 sections. Plants grew for 21 days in soilless mix Fafard 3B (Canadian sphagnum peat moss, 7.62/20.32 cm processed pine bark, perlite, vermiculite, wetting agent, starter fertilizer and dolomitic limestone; Sun Gro Horticulture, Agawam, MA, USA) in the greenhouse (latitude = 34.67350, and longitude = −82.83261) with mist frequency of 8 s every 10 min during the daylight hours. The trays were moved one position in series around the mist bed, shifting daily to compensate for non-uniform irrigation, wind and shading on the greenhouse mist bench (Fig. 1).

Greenhouse growth

After a 21 days acclimatization period, harvested plants were washed free of the soilless mix and dried on paper towels. The relative fresh biomass gain (RFBG) was calculated for the harvested plants/vessel as *Fresh biomass after 21* days *out/Fresh biomass at day 0 in the greenhouse.*

The shoot length and leaf area were measured from flat images using Image J software (Image J/FIJI 1.46, Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). A leaf area index (LAI) was determined from each vessel by dividing the number of initial buds in a vessel into the sum total leaf area of all plants in the vessel (cm^2/bud). The average shoot length for each vessel was determined for shoots above a 4 cm height.

Response surface methods design and statistical analysis

D-optimal criteria were used to select 25 treatments (out of 81 possible combinations of four factors at three levels each). This set of treatment combinations was duplicated to examine an extra qualitative factor (genotypes) at two levels. The factor terms to include in the response surface models (Tables 2, 3, 4) were selected by forward stepwise methods when $P < 0.05$. Statistical criteria including R^2, the adjusted R^2 (R_a^2), the predicted R^2 (R_p^2), the overall F *statistic*, and *P* value, were used to assess the utility of each model. The MS control experiment was run concurrently to test the effects of two genotypes and the three levels of

Table 1 The twenty-five treatments combinations of four factors, in vitro plant density (buds/L), PO_4^{3-} mM, KNO_3 mM, and Ca^{2+} mM and SO_4^{2-} mM was a covariate

Treatments	Buds/L	PO_4^{3-} mM	KNO_3 mM	Ca^{2+} mM	SO_4^{2-} mM
1	50	10.25	18	2	1.5
2	50	10.25	100	10	8.25
3	50	6.25	18	10	10.25
4	250	10.25	100	2	1.5
5	250	6.25	100	10	10.25
6	50	6.25	100	10	10.25
7	50	6.25	18	2	2.25
8	150	6.25	100	6	6.25
9	250	10.25	100	10	8.25
10	250	6.25	18	2	2.25
11	250	6.25	18	10	10.25
12	50	10.25	18	10	8.25
13	150	8.25	59	2	2.5
14	50	10.25	59	6	4.25
15	150	8.25	100	10	9.25
16	150	10.25	59	10	8.25
17	50	8.25	18	6	5.25
18	250	8.25	59	6	5.25
19	250	6.25	100	2	2.25
20	50	10.25	100	2	1.5
21	50	6.25	100	2	2.25
22	250	10.25	18	2	1.5
23	50	6.25	59	10	10.25
24	150	6.25	18	6	6.25
25	250	10.25	18	10	8.25

The entire design was repeated for two genotypes of *Curcuma longa* genotypes L35-1 and L22-5

Fig. 1 Daily movements of trays distributed error due to light and misting irregularities in the greenhouse

plant densities in the basal MS medium (1962) in a completely randomized experiment (2 × 3 factorial arrangement with the three replicates). The factorial model terms were considered significant at $P < 0.05$. The experimental designs, analysis, and graphs were created using JMP version 11.0 (SAS Inst., Cary, NC, USA).

Table 2 Turmeric relative fresh biomass gain (RFBG) was affected by in vitro plant density, KNO_3, and Ca^{2+} after 21 days acclimatization period

Model terms	Model coefficients	*P* value	Mean square
KNO_3 mM	−0.4637 ± 0.0990	<0.0001	5.7488
Buds/L × KNO_3 mM	0.3604 ± 0.1139	0.0036	2.6280
KNO_3 mM × Ca^{2+} mM	0.2697 ± 0.1121	0.0228	1.5187
(KNO_3 mM)^2	−0.4609 ± 0.2101	0.0364	1.2640
Buds/L	0.0587 ± 0.1052	0.5812	0.0816
Ca^{2+} mM	−0.0509 ± 0.1013	0.6186	0.0664

The forward stepwise method selected the final model that has $R^2 = 0.582$, $R_a^2 = 0.496$, and $R_p^2 = 0.313$, F *statistic* = 6.748 at $P < 0.0001$

Table 3 Turmeric leaf area index (LAI) produced after 21 days acclimatization period was affected by in vitro plant density, KNO_3, Ca^{2+}, and genotypes

Model terms	Model coefficients	*P* value	Mean square
(KNO_3 mM)^2	−22.214 ± 6.8648	0.0031	2823.9931
Buds/L × KNO_3 mM	10.7134 ± 3.7693	0.0083	2178.5633
Ca^{2+} mM	−7.1577 ± 3.3030	0.0389	1266.5633
Buds/L	−6.0442 ± 3.3828	0.0848	860.9083
Genotype L22-5	−3.4981 ± 2.8148	0.2243	416.4939
KNO_3 mM	1.7429 ± 3.2932	0.6008	75.5367

The forward stepwise method selected the final model that has $R^2 = 0.502$, $R_a^2 = 0.396$, $R_p^2 = 0.146$, and F *statistic* = 11.2404 at $P < 0.0068$

Results and discussion

Relative fresh biomass gain (RFBG)

During the 21 days acclimatization period, fresh biomass from plantlets grown on basal MS medium more than doubled (2.6 ± 0.5 fold for both genotypes), regardless of plant density. In treatment media, subsequent RFBG could be optimized by selecting KNO_3 concentration in the tested range, as indicated by the significant negative quadratic term (Table 2). The interaction of KNO_3 with plant density and with Ca^{2+} significantly affected the subsequent RFBG during the 21 days acclimatization period of both genotypes (Table 2). Plantlets grown at high plant density required 42 mM KNO_3 and 2 mM Ca^{2+} in vitro to increase subsequent RFBG 3.3 ± 0.5 fold. Plantlets grown at low plant density and subjected to 18 mM KNO_3 and 2 mM Ca^{2+} in vitro resulted in the greatest subsequent RFBG (3.7 ± 0.5 fold, Fig. 2). Choosing KNO_3 as a macronutrient salt to charge balance NO_3^- made it difficult to distinguish between the effects of NO_3^- and K^+. Although K^+ and NO_3^- were supplied in equimolar amounts, spent medium analysis showed twice as much K^+ remained following the culture period (Fig. 3). This suggests that regardless of NO_3^- concentration, the concentration of K^+ was more than sufficient for maximum growth, and the increased demand for KNO_3 in high-density culture was driven by the increased need for NO_3^-.

In a similar study, turmeric subsequent RFBG after a 14 days acclimatization period had been affected by mineral nutrition in vitro, but the non-uniformity in mist bed

Table 4 Turmeric shoot length was affected by in vitro plant density, PO_4^{3-}, KNO_3, Ca^{2+}, and genotypes after 21 days acclimatization period

Model terms	Model coefficients	*P* value	Mean square
Buds/L	−0.7432 ± 0.2001	<0.0012	11.2099
(KNO_3 mM)^2	−1.4132 ± 0.3959	0.0017	10.3582
KNO_3 mM	0.4458 ± 0.2015	0.0376	3.9810
Ca^{2+} mM	−0.4034 ± 0.1942	0.0497	3.5068
Buds/L × PO_4^{3-} mM	0.4050 ± 0.2012	0.0565	3.2962
Genotype L22-5	0.3095 ± 0.1622	0.0695	2.9610
KNO_3 mM × Ca^{2+} mM	0.4088 ± 0.2217	0.0787	2.7646
Genotype L22-5 × KNO_3 mM	0.3423 ± 0.1825	0.0851	2.6440
(Buds/L)^2	0.6379 ± 0.3952	0.1207	2.1188
Genotype L22-5 × PO_4^{3-} mM	−0.2693 ± 0.1825	0.1542	1.7707
PO_4^{3-} mM	−0.2805 ± 0.1959	0.1662	1.6677

The forward stepwise method selected the final model that has $R^2 = 0.727$, $R_a^2 = 0.591$, and $R_p^2 = 0.311$, and F *statistic* = 5.3413 at $P < 0.0004$

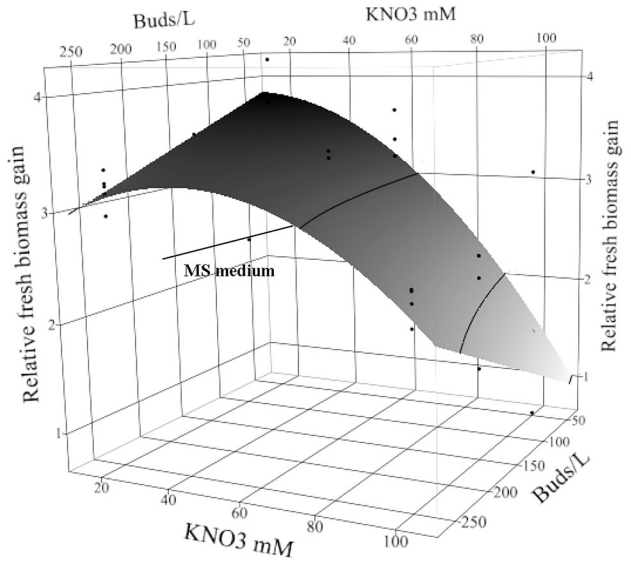

Fig. 2 Effect of in vitro plant density and KNO_3 concentration on the relative fresh biomass gain of turmeric after 21 days acclimatization period. The counter surface and the surface plus residual points were presented when Ca^{2+} concentration was set at 2 mM. The *line segment* shows MS relative fresh biomass gain plotted to 40 mM NO_3^-

conditions, wind, light intensity, and irrigation, may have caused uncontrolled error that resulted in the model having low reliability and predictability (Adelberg et al. 2013). Water loss from the leaf surface during acclimatization severely limits growth, and controlling relative humidity, air movement and light intensity in the acclimatization environment is necessary to allow in vitro plantlets to grow well in a greenhouse (Kozai 1991). In the present study, the variation in light, wind, and irrigation water on the greenhouse bench was randomly distributed across treatments by rotating the trays in the greenhouse one position every day.

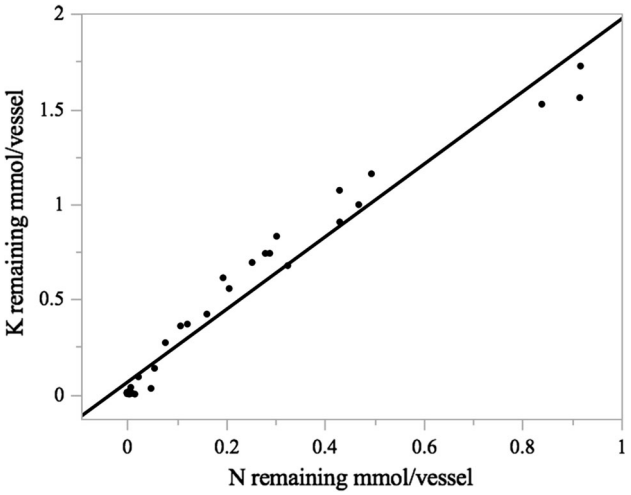

Fig. 3 A regression plot shows the concentration of K^+ and N (mmol/vessel) in spent media after 35 days of turmeric micropropagation. The regression equation is K^+ in remaining media = 0.062659 + 1.9076067 × N in remaining media was evaluated with $R^2 = 0.958$

Leaf area index (LAI)

In vitro plant density significantly affected subsequent LAI of plantlets from MS after the 21 days acclimatization period. Both genotypes had 22 ± 8 cm^2/bud from the high plant density, which was significantly lower than the subsequent LAI from the low plant density (36 ± 8 cm^2/bud). In the treatment media, the significant negative quadratic term and the interaction of plant density with KNO_3 indicated that the optimal concentration of KNO_3 was in the range of the experiment and was affected by plant density (Table 3). After 21 days acclimatization, the treatment media level of Ca^{2+} significantly affected subsequent turmeric LAI (Table 3) in both genotypes. In vitro medium with 70.4 mM KNO_3 and 2 mM Ca^{2+} and at high plant density resulted in subsequent turmeric LAI of 67 ± 16.4 cm^2/bud. In comparison, treatment medium with 50.7 mM KNO_3 regardless of Ca^{2+} and at low plant density, maximized subsequent turmeric LAI (78.4 ± 16.4 cm^2/bud) (Fig. 4). In the optimal media, N concentration was still in a similar range to that found in MS medium except NO_3^- was higher in the optimal media since NH_4^+ had been reduced from 20 mM to 5 mM, and K^+ was used as a counter ion instead of NH_4^+ or Na^+. Leaf area and shoot length of field grown turmeric correlated well with final rhizome yield (Panja et al. 2002; Ravindran et al. 2007 and citation within).

Shoot length

Genotypes grown on MS medium had different shoot lengths. Genotype L22-5 elongated to 6.0 ± 0.6 cm regardless of plant density after 21 days acclimatization. Shoot length of genotype L35-1 elongated to 4.0 ± 0.6 cm from high plant density and to 6.0 ± 0.6 cm from low plant density. Similar to RFBG and LAI from the treatment media, the subsequent turmeric shoot length could be optimized by selecting KNO_3 in the tested range as indicated by the significant negative quadratic term (Table 4). Plant density and Ca^{2+} significantly affected the shoot length regardless of the genotype (Table 4). The RSM identified that high plant density growth with medium containing 64 mM KNO_3, 6.25 mM PO_4^{3-}, and 2 mM Ca^{2+} resulted in shoot length of 8.0 ± 0.9 cm after a 21 days acclimatization period, which was shorter than plants from low plant density in the same medium (10.9 ± 0.9 cm, Fig. 5). The negative quadratic term of KNO_3 indicates that the optimal concentration is in the

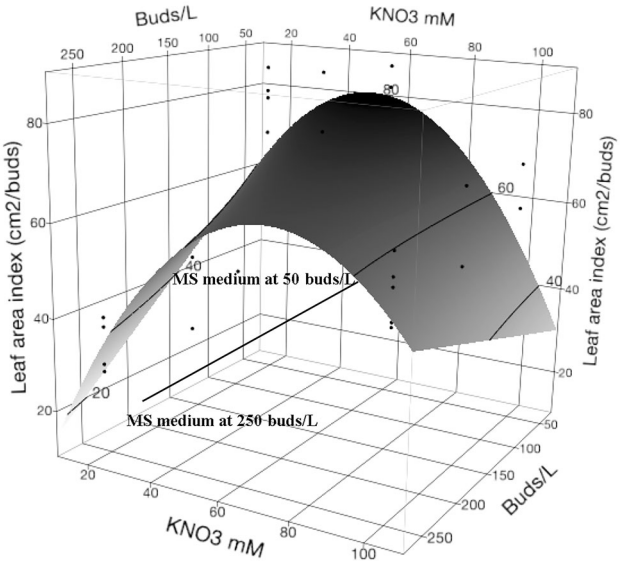

Fig. 4 Effect of in vitro plant density and KNO_3 concentration on the leaf area index (cm^2/bud) of turmeric after 21 days acclimatization period. The counter surface and the surface plus residual points were presented when Ca^{2+} concentration was set at 2 mM in vitro. The *line segment* shows the leaf area index of plants from MS at high and low plant density plotted to 40 mM NO_3^-

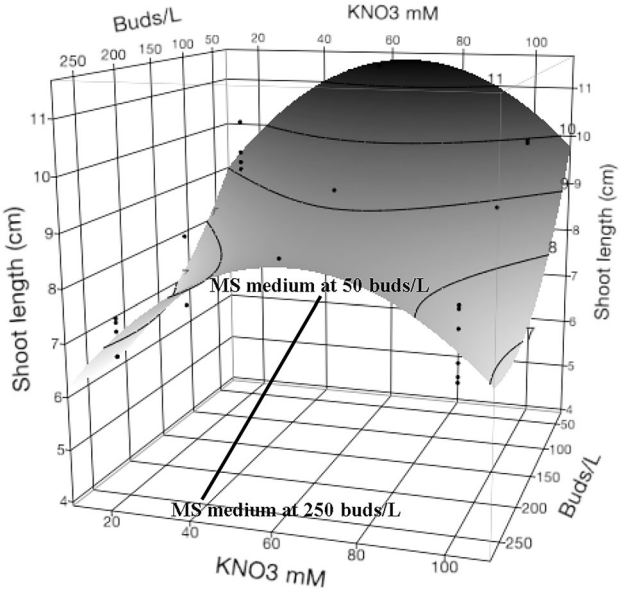

Fig. 5 Effect of in vitro plant density and KNO_3 concentration on the shoot length (cm) of turmeric after 21 days acclimatization period. The counter surface and the surface plus residual points were presented when other in vitro factors were set at 6.25 mM PO_4^{3-} and 2 mM Ca^{2+}. The *line segment* shows shoots length from MS plants in low and high plant density plotted to 40 mM NO_3^-

range that was tested. In the present study, the optimal in vitro treatment concentration of K^+ was 3–4× greater and NO_3^- was 1.5× greater than concentrations specified in MS.

With proper combination of factors, plants from treatment media were larger 21 days after acclimatization than those grown in MS (Fig. 6). The differences in RFBG, LAI, and shoot length were plainly observed when viewing the young plants. Many scientists have used the half MS medium for the third stage of micropropagation (shoot development) to avoid the high N concentration in the medium (especially NH_4^+) that may cause hyperhydricity (Ziv 1991; George et al. 2008; Ivanova and Van Staden 2009; Reed et al. 2013b) and to improve in vitro rooting of transferring plants (Moncousin 1991; Economou 2013). However, diluting medium concentration could result in inadequate concentrations of other biologically significant minerals (Adelberg et al. 2013).

The concentration of Ca^{2+} in the optimal media (2 mM) was lower than the concentration specified in MS (3 mM). In the cytosol, free Ca^{2+} is a signal transducer for many enzymes. Thus Ca^{2+} concentration is highly regulated and is transported out of the cytosol by energy-dependent efflux (Taiz and Zeiger 2006). In the treatment media, the uptake of Ca^{2+} and plant tissue Ca^{2+} concentrations increased with increasing Ca^{2+} concentration (data not shown). During acclimatization, the photosynthetic process might not provide adequate energy to maintain efflux of Ca^{2+} from the cytosol. Reducing the strength of MS medium to half might enhance the subsequent acclimatization growth of in vitro plants with lowered $Ca^{2+}$and less NH_4^+, but more KNO_3 and PO_4^{3-} are needed for stage III pre-hardening.

Conclusion

In the present study, KNO_3 was the most important nutrient factor that increased subsequent *ex vitro* growth from high plant density culture. The residual K^+ in the remaining media indicated that the supply of K^+ in the higher concentrations might not be necessary as a nutrient and was tolerated by rapidly growing plants at high density. Potassium cation was useful for increasing NO_3^- in a salt-based formulation with reduced NH_4^+. Murashige and Skoog (1962) relied on NH_4NO_3 to provide one-third of the NO_3^- concentration.

A low NH_4^+ (5 mM) stage III medium that was developed in prior works to promote turmeric growth during acclimatization that also had a high PO_4^{3-} concentration (Adelberg et al. 2013; El-Hawaz et al. 2015a). In this study, the PO_4^{3-} concentration tested were 5× to 10× greater than the 1.25 mM PO_4^{3-} used in basal MS medium. The fact that PO_4^{3-} did not influence *ex vitro* quality metrics, suggests that 6.25 mM PO_4^{3-} is adequate for turmeric during

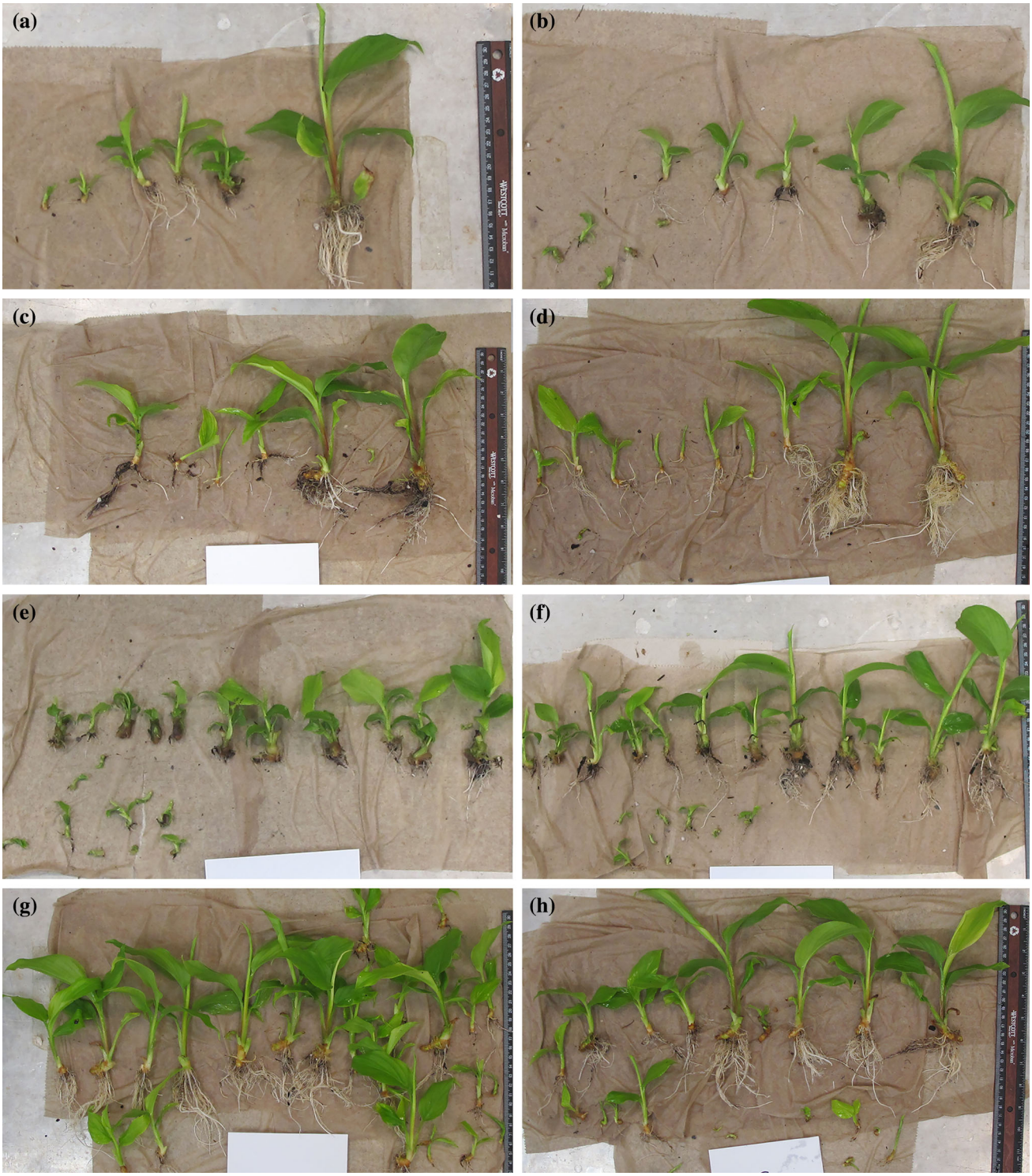

Fig. 6 Two-dimensional images of *Curcuma longa* after 21 days of *ex vitro* growth to show the effect of in vitro growth media. Low plant density on MS medium **a** genotype L35-1 and **b** genotype L22-5 compared to plants grown at low plant density, 10.25 mM PO_4^{3-}, 2 mM Ca^{2+}, and 59 mM KNO_3, **c** genotype L35-1 and **d** genotype L22-5. Plants grown in vitro on MS medium at high plant density, **e** genotype 35-1 and **f** genotype L22-5 compared to the treatment of high plant density, 10.25 mM PO_4^{3-}, 10 mM Ca^{2+}, and 100 mM KNO_3, **g** genotype L35-1 and **h** genotype L22-5. As a consequence of using D-optimal selection, less than one-third (25/81) of the treatment points were tested thus treatment points photographed here were closest to the treatment *highlighted*

subsequent acclimatization. Turmeric grew larger and faster during the *ex vitro* acclimatization period when subjected to high PO_4^{3-}, high KNO_3, low Ca^{2+} and low NH_4^+ media in comparison to the MS medium. The optimal concentrations depended on the density of plants in culture, with greater densities preferred for commercial application and lesser density yielding the largest plants.

In vitro mineral nutrition is a critical factor for the subsequent acclimatization stage before the root system is established in the soilless mix. Selecting an optimal media requires defining a high or low density application, and choosing which *ex vitro* responses are most important. Only after these decisions are made, can a nutrient medium be formulated from inorganic salts.

Using RSM allows for modification of multiple factors within in vitro media for better *ex vitro* growth. In a plant propagation nursery, once the plant has attained the proper size, it is removed from the subsequent mist and repotted to a larger container. The rapid removal of acclimatized plants under mist reduces the risk of pest infestation and allows an early application of conventional fertilizer, therefore increases the efficiency of the propagation nursery. In vitro treatments that make *ex vitro* plants grow more quickly through acclimatization are superior as propagation stock.

Acknowledgments We are thankful to Dr. Kathy Moore of the Clemson University Agricultural Service Laboratory for sample analysis and to Mr. Fatih Bensriati for helping with data collection. We also acknowledge the Libyan Ministry of Education and Scientific Research for the graduate stipend of the senior author.

Authors' contribution RE: The corresponding author, a graduate student who designed and conducted the experiment and done the lab and greenhouse work as well data analysis. DP: The professor who assisted with reviewing and editing the paper assisted primary author on understanding the *ex vitro* importance and morphological growth responses of different minerals utilized in in vitro medium. WB: The professor who assisted with the statistical design and data analysis. JA: The advisor professor where all the work done in his lab and under his advice also he assisted with data collecting.

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