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SIMULTANEOUS OPTIMIZATION OF MACRONUTRIENT FACTORS IN PLANT TISSUE CULTURE USING TURMERIC (Curcuma longa L.) AS A MODEL

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ABSTRACT

Murashige and Skoog 1962 performed one-factor-at-a-time (OFAT) experimentation in order to regenerate tobacco callus on semi-solid agar medium. This work became an established medium for tissue culture micropropagation and experimentation. Micropropagation is done in niche markets with herbaceous perennials, among other crops, and the optimization of various inputs to produce maximal responses is a necessary step towards process development. This current study of macronutrient factors simultaneously altered media volume, amount of tissue (plants per vessel), sucrose, nitrogen (as NO_3^- and NH_4^+ ions), and K^+ in a d-optimal design space with only 55 experimental units (including 5 true replicates). The first study examined these macronutrients in a micronutrient limited environment, and probed further areas in the design space for exploration. The second study, at full MS meso- and micronutrient values, identified P and Mg to be deficient in standard formulations when compared with field-grown plantlets of turmeric, while identifying differences in the definition of plantlet quality. Plantlet quality was defined in three very distinct ways. These three optimization choices were demonstrated to have very different optima as defined in this experiment. First: multiplication is maximal with low plantlet density (3 plantlets per vessel), high media volume (45 ml), and 4% sucrose in the vessel. Secondly: the number of new plants produced per vessel was highest when the most plants were put in a vessel, at the highest media volume, and highest sucrose % tested. Lastly, those individual plantlets transferred to the greenhouse (100% plantlet survival) which grew the most (via fresh mass gains) were the most massive (fresh mass) came from vessels with: 3 plantlets

per vessel, containing no NH_4^+ (all K^+), 45 ml media volume (the highest amounts of media components holding concentrations constant), and the lowest concentration of sucrose tested (1.5%). These two experiments result in separation of optima demonstrating the need for differing tissue culture medium formulations that are dependent upon the process of interest, while identifying possible areas of future work necessary for in vitro nutritive media formulations in turmeric (*Curcuma longa* L.), an important medicinal herbaceous perennial.

DEDICATION

This thesis, submitted to the Graduate School of Clemson University, would not have been possible without the guidance of Dr. Jeffrey Adelberg. The cooperation and guidance of Dr. Randall Niedz, Dr. Halina Knap, and Dr. William Bridges was integral to the execution of this manuscript, and their input deserves recognition. All experiments carried out in Dr. Adelberg's tissue culture laboratory would not have been possible without the assistance and guidance of Dr. Adelberg, Jackie Naylor-Adelberg, Lauren Duncan, Kenneth Krantz, and Dr. Sun Youping. This manuscript is dedicated to those friends and family who supported the author both financially and intellectually.

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CHAPTER ONE

A MACRONUTRIENT OPTIMIZATION PLATFORM FOR MICROPROPAGATION AND ACCLIMATIZATION: USING TURMERIC (*Curcuma longa* L.) AS A MODEL PLANT

Abstract

Tissue culture media is often overlooked as a factor in plant biotechnology. Most work uses Murashige and Skoog 1962 inorganic media formulation which is not likely optimal for many of the plant systems where it is used. This current study of macronutrient factors simultaneously altered media volume, amount of tissue (plants per vessel), sucrose, nitrogen (as NO_3^- and NH_4^+ ions), and K^+ in a d-optimal design space with only 55 experimental units (including 5 true replicates). Plantlet quality was quantified by multiplication in the laboratory and survival and growth in the greenhouse. With the lowest plant density, the lowest macronutrient concentration (20 mM) and equi-molar proportions of NH_4^+: K, the plantlets had best multiplication ratio and 100% greenhouse survival. Multiplication ratio in vitro and survival in the greenhouse were well correlated with one another. Laboratory dry mass, media use, sucrose use, and the uptake of the macronutrients, NO_3^-, NH_4^+, and K^+ , were not well correlated with plantlet quality. Plantlets with the greatest uptake of P, Ca, Mg, and Mn had the best multiplication in the laboratory and on subsequent transfer, acclimatized and grew fastest in the greenhouse. Phosphorus was shown to be most depleted in media. This work demonstrates a platform to simultaneously optimize several nutritive components of tissue culture media to produce plantlets that perform well in both laboratory and greenhouse environments. Plant quality was related with factors outside the experimental

design and this platform indicated where to expand the experimental space. Fixed, flat screen presentations reveal less of the response surface than interactive profiles driven by the reader.

Introduction

The most commonly used media in both commercial and experimental tissue culture (Murashige and Skoog 1962) was formulated nearly 50 years ago using a one-factor-at-a-time (OFAT) methodology. Murashige and Skoog's work optimized tobacco callus growth on agar-solidified medium by varying salts one at a time until the amount of callus growth was maximized. A primary problem with OFAT experiments is that OFATs cannot identify interactions. OFAT media formulation does not account for mineral nutrient proportions, such as N:P:K common in fertilizer formulation. Furthermore, critical nutrient ratios should be extended to clusters relating macro-, meso-, and micronutrients (Scagel et al. 2008). Many widely used plant tissue culture formulations were also developed via the OFAT approach including media specified for tobacco callus (MS, 1962), isolated cells (Gamborg 1968), woody shrubs (Lloyd and McCown 1980; Anderson 1980), nut and hardwood trees (Driver and Kuniyuki 1984), epiphytic orchids (Knudson 1946; Vacin and Went 1949), protoplasts (Kao and Michayluk 1975), anthers and microspores (Nitsch and Nitsch 1969; Chu et. al 1975). Most in vitro research uses a media from the literature closest to the application.

Another problem in media formulation, recognized by Murashige and Skoog (1962), was that water is likely rate-limiting to growth when a gelling agent like agar is used. In agar, gradients are established and there is preferential binding of nutritive elements and impurities, thus nutrient uptake cannot be optimal in a water-limited system. Numerous researchers have concluded that media components can become rate-limiting when bound in agar based media (Adelberg and Toler 2004; Klimaszewska et al. 2000; Ramage and Williams 2002; Debergh 1983). Liquid medium is preferable for the study of nutrient uptake due to increased water availability, more rapid plant growth, easier processing during media analysis, and the homogeneity of solution available to the plant (Adelberg et al. 2010). Since moving into liquid medium, solutes, not water now become rate limiting.

A frequent approach to improve media composition is to modify an established media or components by supplementing varying concentrations of a salt, dilutions of existing media, additions of hormones, carbohydrate sources, or other nutritive elements in a trial-and-error fashion until sufficient plant responses are demonstrated (Basile et al. 1993, Kretzschmar et al. 2007, Jain et al. 2009). Identifying the specific combinations of ingredients that resulted in optimal growth was unlikely due to the possibility of interactive effects being confounded with main effects.

With over a dozen elements to choose from, few researchers have chosen to go back and take a more comprehensive view of formulation. Fortunately, we are in an era where the availability of specialty software is enabling better design, calculation and visual outputs from more expansive experiments. Our approach to experimental design

for media formulation is 1) selection of the media components potentially important in affecting the selected response(s), and 2) the identification of the growth response(s) to be improved. We hypothesize that some commonly experienced difficulties in greenhouse survival and acclimatization are the result of poor mineral nutrition in tissue from the lab. This has not been approached as an objective of media formulation, even though the greenhouse is where much product value is conferred, or product losses are suffered.

Selection of growth responses (Y's)

Our objective was to determine the "best" conditions for in vitro growth. A more important objective was to observe in vitro conditions that influenced the acclimatization to greenhouse growth. The idea here was that the "best" conditions for in vitro production of plantlets may not be necessarily best for greenhouse acclimatization. To our knowledge, the literature on nutrient medium formulation is lacking responses that are measured after transfer to the greenhouse. To achieve these objectives we measured in vitro responses including multiplication ratio, relative dry mass (dry/fresh), media used, and sucrose used; and greenhouse responses including survival in the greenhouse, plantlet fresh mass and dry mass growth during acclimatization. Also the amount of specifics nutrients from in vitro medium in plantlet tissues during greenhouse acclimatization was correlated with greenhouse performance. That posed the problem of which nutrients to choose in a design, since 15 factors are too many to vary in one experiment.

Selection of media components to vary (X's)

We chose to simultaneously alter the largest and most massive factors in the vessel: media volume (25 to 45 ml), plants (3 to 9 explants), sucrose concentration (1.5 to 6% m/v), macronutrient concentration (20 to 100 mM with $[NO_3^-]= [NH_4^+]+[K^+]$), and cation proportion of $NH_4^+:K^+$ ratio (0 to 0.5). The $NH_4^+:K^+$ ratio and NO_3^- were co-varied, as suggested by Niedz and Evens (2008) since varying the ions in this manner 1) eliminated ion confounding and, 2) maintained electrical neutrality (i.e., all treatments had the same pH without the ion confounding that can occur when pH adjustments are made). The vessel is a closed system with regard to nutrient mass, and the apportionment of nutrients among many growing plants may limit growth. In theory, the amount of tissue chosen effects the amount of a solute needed (sucrose, nitrate, ammonium, and potassium) and therefore we would test if an optimal nutrient concentration would be related to initial density of plant tissue.

These x-factors were arranged as a 5-factor response surface experimental design. Treatment point selection was via d-optimality. The design included 55 treatment combinations: 20 treatments sufficient for fitting a quadratic polynomial model, 30 treatment points for testing lack-of-fit, and 5 replicate treatments to estimate pure error. Geometrically, this 5-dimensional hyper-volume would have a response surface (y) in a 6^{th} dimension that could be described by coordinates of the 5x- factors in areas of interest. This allowed us to simultaneously maximize the main and interactive effects of the x- factors on the measured responses.

We also recognized that when we optimize growth with macro-nutrients, other nutrients not selected would be the limitations to growth. To explore the effects of meso- and micro-nutrients not chosen as x-factors we modified the medium as follows. The amount of P supplied was 100% of MS, while the other meso-nutrients (Ca, Mg), and micronutrients (S, Cl, Fe, B, Zn, Cu, Mn, Mo, Ni, Na) were supplied in very low amounts (5% of MS). This forced deficiencies and enabled a secondary objective: identify other nutrients that were most limiting to growth. By observing how the amount of non-x factor nutrients in the plantlet tissues was correlated with greenhouse performance, allowed selection of critical elements for the next experimental design space.

Materials and Methods

Plant Material

Stage I plantlets of *Curcuma longa* L. accession 35-1 were initiated according to methods described by 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. Full-strength commercial bleach (Clorox™, 5.25% sodium-hypochlorite) was used to disinfect shoot tips for 30 s, they were placed on hood surface to dry, and 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, and 30 g sucrose per liter. Medium pH was adjusted to 5.7, and

Stage I was carried out in 2 ml of liquid medium in flat-bottomed vials. Stage II plantlets obtained from these cultures were maintained for approximately 5 years by subculture prior to experiments described hereafter.

Immediately prior to experimental conditions, Stage II plantlets were multiplied on similar MS media containing MS vitamin stock, 3 μM benzyl adenine, 3% sucrose. Once adequate stock material was prepared, plants were transferred into 55 different treatment combinations (Table 1). Macronutrients were considered as ions, and not salts, to eliminate ion-confounding, and to isolate the specific effects that nutrients have on plant growth in vitro (Niedz and Evens 2006; Evens and Niedz 2008). Balancing the major anion with major cations in media (NO_3^- with NH_4^+, and K^+) and then altering the cation ratios minimized pH problems and nutrient ions were evaluated independently (Niedz and Evens 2004). This design constrained $[NO_3^-]= [NH_4^+]+ [K^+]$.

In vitro experiment

Four, 35 day cycles were run where plantlets were subcultured and returned to same experimental treatment. During subculture, plants were counted, massed, spent media volume determined, and sucrose remaining in media was measured as % BRIX (Atago Model N10, Atago Instruments Ltd.). Cut buds were transferred into fresh experimental media, and a representative tissue sample was taken for dry mass. Plant material for dry mass was dried for 72 hours at 75°C. Spent medium was frozen for analysis at the conclusion of cycle 4.

Spent medium analysis

Medium residual from cycle 4 in vitro was analyzed by Clemson University's Agricultural Chemical Service using a Thermo Jarrell Ash Model 61E Inductively Coupled Plasma (ICP). Nutrient residual was calculated by ICP concentration determination multiplied by volume of spent medium.

Greenhouse experiment

The fourth cycle was the terminal cycle in vitro and the majority of the tissue went into the greenhouse for acclimatization (and a portion of the plant material went for dry mass). Plantlets were taken from vessels, massed, and rinsed before being placed in individual cells on greenhouse mist bed in Fafard 3-B soilless mix (which contains: Canadian sphagnum peat moss, processed pine bark, perlite, vermiculite, starter nutrients, wetting agent, dolomitic limestone, pH 5.5-6.6; Fafard Co., Anderson, SC). After 10 days in greenhouse conditions (Latitude = 34.67350, Longitude = -82.83261; 60% Shade cloth; 6 second on, every 16 min mist cycle during daylight hours; March 14-23) plants were harvested, roots were washed clean of soil, massed again and dried.

Calculated Responses

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

Multiplication ratio $= (\frac{plantlets\ out\ per\ vessel}{plantlets\ in\ per\ vessel})$ is a standard measurement to determine the relative level of multiplication in a fixed time period (35 day cycle).

Percentage dry mass (in the laboratory) $= (\frac{dry\ mass\ of\ sampled}{fresh\ mass\ of\ sampled})$ is a common indicator of lab plantlet quality for subsequent acclimatization and greenhouse survival.

Media use

$= Original\ mass\ of\ vessel\ without\ tissue - (mass\ of vessel\ out\ of\ cycle - fresh\ mass\ of\ plantlets)$

Media use is a summation of water used and solutes used.

Sugar used (%) $= \frac{\%\ Sucrose\ Initial - \%\ Sucrose\ out}{\%\ Sucrose\ Initial}$ shows how much sucrose became dry mass, with the remainder respired for metabolic energy.

Survival in the greenhouse $= \frac{plantlets\ day\ 10}{plantlets\ day\ 0}$, calculated as plants in greenhouse on day 10 (per vessel) divided by plantlets brought to greenhouse at day 0 (per vessel).

Fresh mass change in the greenhouse

$$= (fresh\ mass\ day\ 10) - (fresh\ mass\ day\ 0)$$

Dry mass change in the greenhouse

$$= (dry\ mass\ day\ 10) - (dry\ mass\ day\ 0)$$

Fresh and dry mass change in the greenhouse provide a measure of plantlet quality specific to this stage of development.

The amount of x-factor nutrient ions used by plant: NH_4^+, K^+, and NO_3^-, was expressed as:

NH_4^+, NO_3^-, and K^+ in grams used = (initial grams NH_4^+, NO_3^-, and K^+) X [1- NH_4^+, NO_3^-, and K^+ ppm in spent medium X Media Out (ml)/(NH_4^+, NO_3^-, and K^+ppm initial X Media Initial (ml)

The amount of non-x nutrient ions used by plant: P, Ca, Mg, Zn, Cu, Mn, Fe, S, Na, B, and Cl, was expressed as a percentage of total supplied:

$$P, Mg, Mn, or\ Ca\ \%\ \text{used} = \left[1 - \frac{P,Mg,Mn,Ca\ ppm\ in\ spent\ medium \times Media\ Out\ (ml)}{P,Mg,Mn,Ca\ ppm\ initial \times Media\ Initial\ (ml)}\right]$$

We chose to present 14 of the 23 Y's we measured or calculated due to model fit (data not shown) and relevance to the community.

Statistical design and analysis of the experiment

This experiment was a response surface of four factors at three levels per factor, and one mixture response factor at 4 levels. The factors altered here were: media volume (25, 35, 45 ml per vessel), plantlet density (3, 6, 9 plantlets per vessel), sucrose concentration (1.5, 3.75, 6 % sucrose m/v), NO_3^- ion concentration (10,30,50 mM), and NH_4^+:K^+ ratio (0:1, 0.5:0.5, 0.25:0.75. 0.125:0.875) with total mM of cations equivalent to NO_3^-. Factor combinations were assigned to 55 experimental units (in vitro vessels) according to a d-optimality criterion, including 5 true replicates (Table 1). This allowed resolution of quadratic terms, evaluation of 2^{nd} order interactive effects between factors (X's), but confounded higher order interactive effects. The model proposed to relate factors to responses was a response surface model. A significance level of 0.05 was chosen for all tests. Multivariate correlations among responses were considered "well correlated" where Pearson's $r > 0.60$. Design, analysis, and graphical visualizations were created using JMP 9.0 (SAS Inst. Cary, NC).

Results and Discussion

Four subculture cycles in vitro were carried out in tandem so plantlets approached a steady state with treatment conditions. Cycle 1 was an inductive phase when the nutrient status in each plantlet became more reflective of treatment conditions and less like tissues from MS 1962 media (due to nutrient carryover in sub-cultured bud and

microrhizome). Cycles 2, 3, and 4 better approached stable growth and development in treatment conditions. Cycle 4 was the final cycle where medium was analyzed and plants were transferred to greenhouse. Our first objective focused on multiplication ratio and greenhouse survival since we consider these to be important responses in micropropagation. Different physiological conditions that may confer plantlet quality become apparent when correlating measured responses in laboratory to subsequent events in greenhouse.

Multiplication ratio

The analysis of multiplication ratio over the four culture cycles showed a decrease following the first cycle, when the plantlets adjusted to the 5% meso-/micronutrient limitation (Figure 1; Table 2). The response with the 5 independent variables analyzed over cycle produced a model with highly significant terms (0.0001<"Prob>|t|">0.001), moderately significant terms (0.001<"Prob>|t|">0.05), and insignificant terms ("Prob>|t|"<0.05) as shown in Table 2 (we will not present insignificant terms in any of the models shown later in this paper). The *sorted term estimates* quantify the predicted effect of each independent variable, or term (Estimate column); evaluate the error associated with that prediction (Std. error); evaluate magnitude (direction + quantity) of independent variable effect on the response (t Ratio = estimate/standard error); and evaluate the probability that the independent variable (x) has no effect on the response (Prob>|t|). In the lower portion of Table 2, the *Model Fit Analysis* outlines several ways

to evaluate the model fit: *whole model hypothesis test* if a model fits the data better than the default model (the treatment mean response line) using an F ratio and p-value (Prob>F), *lack of fit hypothesis test* if the form of the model is adequate for the data, the *error standard deviation* shows the mean of the squared errors produced by the model (this number should be low), and the *RSquare* indicates how much of the variation in the response is explained by the model fit. For example, 0.5778 = 57.78% of variation in response is explained by the model shown in Table 2.

Table 2 shows the effect of x-factors on multiplication. Increased Macronutrients (mM) negatively affects multiplication given the negative estimate, small error, negative t Ratio, and extremely low p-value which means the effect of macronutrients on multiplication is not zero. Also, NH_4^+:K^+ ratio (2^{nd} most influential model term) positively affects multiplication, so with added NH_4^+ with equi-molar reduction in K^+, multiplication increased since the estimate is positive, the error around that estimate is relatively small, the t ratio is positive, and the extremely low p-value tells us that the effect of NH_4^+:K^+ ratio is not zero. The Plants/Vessel term also had a negative effect on multiplication and more plants per vessel caused a limitation to multiplication. The $Cycle^2$ term indicated that multiplication ratio from cycle 1-4 was a quadratic function and plantlets had not adjusted to treatment conditions during cycle 1. Cycle interacted with Macronutrient concentration. We were able to disregard interactive effects of nutrients and cycle because the interactive effects are only significant with cycle 1 in model. When cycles 2, 3 and 4 were analyzed, the interaction of Macronutrient concentrations with cycle time was not significant (data not shown). Therefore cycle 1

was correctly considered and inductive period, and conclusions made for cycle 4 will be more representative of other cycles at stasis with treatment conditions.

To simplify the view of how cycle affects the response, multiplication was presented (Figure 1). Mean response from cycle 1 (initiation) was significantly higher than the mean responses generated from cycles 2 – 4. The differences between cycles 2-4 were much smaller than their differences with cycle 1. Multiplication was greatest in those vessels with lowest macronutrient concentration, fewest plants per vessel, and equi-molar NH_4^+:K^+ ratio (the highest ratio tested), but this cannot be shown in a simple figure.

Multiplication Ratio Cycle 4

We chose to model of multiplication ratio in cycle 4 and it was used in correlational analysis of laboratory growth and greenhouse plantlet quality. Multiplication in cycle 4 was well described by the x-factors in this experiment (Table 3, RSquare = 0.7634). Table 3 showed increased Macronutrient concentration (mM) had a negative effect on multiplication (as we saw in model fit of all four cycles); there was a significant quadratic NH_4^+:K^+ ratio^2, increased plants per vessel negatively effects multiplication, and the NH_4^+:K^+ ratio interactions with plants per vessel and media volume were significant, as well as, the significant main effect of NH_4^+:K^+ ratio. Visualizing complex responses to multifactor experiments requires some explanation (Figure 2a). When interaction was insignificant, the line traces that defined the

boundaries of the response surface were nearly equi-distant or collinear, and the response surface that bounds the hyper-volume was smooth in those dimensions. The paired main effects can be visually interpolated at a point of interest by the unaided eye.

However, the colored blocks in Figure 2a showed many pairs of surface traces that had boundary traces of varied distances, indicating the response surface twisted in those dimensions. For example, the blue box labeled i in Figure 2a shows the surfaces created by NH_4^+:K^+ ratio are quadratic terms, visualized by curved line traces. When NH_4^+:K^+ was lowest, the plantlets multiplied slowly regardless of plantlet density. The interaction of NH_4^+:K^+ ratio with Plants/Vessel showed maximized multiplication with 3 plants vessels and the greatest NH_4^+: K^+ ratio. When there were 9 plants in the vessel, the response of NH_4^+:K^+ was flatter, showing a lesser effect on multiplication, and with the entire surface pulled downward (reduced multiplication) much more sharply at higher 0.5 NH_4^+: 0.5 K^+ ratio, than if NH_4^+ was absent. The blue box labeled ii shows how plants per vessel bounded the same surface. The quadratic response to NH_4^+:K^+ ratio also suggested that there is little advantage to going higher than 0.5 NH_4^+: 0.5 K^+ ratio. Each interactive effect can be visualized using this format. However, visualizing pairs of interactive x-factors misses the power of optimizing responses with multiple variables in a single design (5 factors in our design). Other factor pairs in this matrix also drive this response. This response surface exists in 6-dimensions and the human eye (even when aided by the creative mind) will find it difficult to identify a maximal effect by integrating and interpolating the curves, slopes, and twists of these unseen surfaces.

A simpler way to work with response surfaces is to compare responses at fixed points using a *response surface prediction profiler.* The profiler allows 1) the user to set x-coordinates and predicts response, or 2) solves for x-coordinates at a selected response, using a model that simultaneously adjusts for the main and interactive terms. To demonstrate this capability, we choose x-coordinates to be "typical" of standard MS culture conditions and predict the response (y). Then we maximize for the response (y) and show the x-coordinates. Comparing these two sets of x-coordinates will show how far the standard medium was from optimal conditions for each response. MS media macronutrients were not included in this design, but there was an interpolated multiplication ratio of 1.8x at MS macronutrient levels (and standard culture conditions for this vessel size; NH_4^+:K^+ ratio=0.5:0.5, Macros=80mM, %Sucrose=3%,media volume=33ml, plants per vessel=6) (Figure 2b). Multiplication ratio increased to approximately 3.7X by reducing the macronutrient concentration to 20mM, and reducing the number of plants per vessel to 3 (Figure 2c). Macronutrient concentrations were inter-related to explant density and to achieve high performance multiplication medium for lower plant densities required nutrient balance that was not as effective in higher density cultures. In commercial practice, larger numbers of plants per vessel are used to increase the total yield (multiplication x plants per vessel) and there may be less demand for an optimal formulation. Using a dynamic profiler (Appendix I), each reader can explore other regions of the response surface and better understand the relationship between multiplication, yield and the x-factors used in this design.

Reducing the amount of NO_3^-, NH_4^+, and K^+ while changing the ratio of these ions to roughly 4:3:1 respectively, appears to maximize multiplication at low explant densities. This illustrates that adjustments to macronutrient components of tissue culture media greatly affect multiplication ratio and if dilutions are made of MS, it cannot affect ion proportions which were demonstrated to be important. Having reduced meso- and micro-nutrients to 5% of MS (with only P at 100% MS) created a proportion shift where the three macronutrient ions in this experiment were optimal at lowered concentrations. Sucrose and media volume, the largest mass terms and had no significant effect on multiplication ratio (Table 3).

Greenhouse Survival and Growth

Survival of plantlets during greenhouse transition phase was largely dependent upon the concentration of macronutrients in the laboratory, and the interaction of macronutrient ratio and density of plants per vessel (Table 4). With factor coordinates set to MS macronutrients (NH_4^+:K^+ ratio =0.5:0.5, Macros=80mM, %Sucrose=3%) and other typical conditions for this vessel size (media volume=33ml, plants per vessel=6); 77% of the plantlets survived transfer to the greenhouse (Figure 3a). By altering the NH_4^+:K^+ ratio from MS 0.5 NH_4^+: 0.5 K^+ ratio to 0.25 $NH_4^{+\,+}$: 0.75 K^+ and reducing the amount of macronutrients by 50% (Figure 3b), plantlet loss was nearly eliminated and greenhouse survival approached 100%. Plantlet density interacted with NH_4^+:K^+ ratio, such that when

3 plants in the vessel, the balance of macronutrients is more important to survival than with 9 plants in the vessel.

Fresh mass gain during acclimatization in the greenhouse (table 4) showed that reduced macronutrients increased subsequent greenhouse fresh mass gain. When sucrose was increased in the laboratory medium the fresh mass gain increased in the greenhouse. Possibly sucrose created more negative osmotic potentials, or other energy-related terms, that drove growth in the greenhouse. Fresh mass gain in the greenhouse was greatest at 3 plants/vessel, 20 mM macronutrients, 6% sucrose, and 0.5 NH_4^+:0.5K^+ ratio.

For both survival and fresh mass gain, MS macronutrients were far too concentrated with the reduced meso- and micronutrients used in this experiment. Ratios of nutrients in media are more important than amount, and cannot be achieved by simple dilutions of MS, or other standard medium. The sensitivity to macronutrient proportion and concentration is more pronounced at low plant density for both multiplication and greenhouse growth.

Spent Medium Analysis

Phosphorus, supplied at 100% MS level (1.25mM), was the most depleted element, with 66% of the vessels having less than 10% of initial P remaining in the vessel at the conclusion of the fourth cycle. Phosphorus use was largely dependent upon the ratio of NH_4^+:K^+, suggesting the ratio of N:P:K is not only important in agronomy, but also plant tissue culture (Table 6). Less P is taken up by the plant when there is more K^+

than NH_4^+, and less P is taken up in the presence of high concentrations of the macronutrients. Increased plants per vessel increased the percentage P used. Greater media volume increased the amount of P supplied (along with N,K^+) and decreased the percentage P used. This alerts us to the importance of N:P:K in plant tissue culture.

The use of P is related to multiplication ratio and survival in the greenhouse. At standard MS conditions (NH_4^+:K^+ ratio=0.5:0.5, Macros=80mM, %Sucrose=3%,media volume=33ml, plants per vessel=6), when survival was approximately 80%, multiplication is approximately 1.8X, and about 80% of the P was used (Figure 4a). Maximal multiplication and greenhouse survival (Figure 4b), occurs where NH_4^+:K^+ ratio=0.35:0.65 , macronutrients = 20mM, and 3 plants/vessel; however, this prediction requires more than 100% of the P supplied. Phosphorus is likely a limitation to plant performance, and it is possible that nutrient uptake becomes more difficult as the element becomes more diffuse in media.

Similarly, the simultaneous prediction profiler of P, Ca, and Mg use during laboratory cycle 4 (expressed as % use of total in vessel; Figure 5) was shown along with multiplication ratio and survival in the greenhouse. Phosphorus was provided at 100% of MS concentration (1.25mM), while Ca and Mg were provided at 5% of MS concentration (0.15mM, 0.075mM respectively) and we expected to find rapid depletion of Ca and Mg. However, at MS standard levels (NH_4^+:K^+ ratio=0.5:0.5, Macros=80μM, %Sucrose=3%,media volume=33ml, plants per vessel=6) approximately half of Ca remained, 34% of Mg remained, and only 17% of P remained, where survival was 80% and multiplication ratio was 1.8X (Figure 5a). With macronutrients “out of balance”

meso-nutrient uptake was low. Figure 5b shows that reducing the NH_4^+:K^+ ratio to 0.25:0.75 and total macronutrients to 20mM; P, Ca, and Mg are completely depleted in medium, multiplication ratio is 3X, and there was 100% survival in greenhouse. With macronutrient elemental ratios "corrected", these mesonutrients were depleted from the medium and plantlet quality improved.

When we maximize multiplication ratio or survival in the greenhouse, P, Ca, and Mg quickly become limited in the media. The reader can explore the loss of meso-nutrients during the simultaneous maximization of multiplication and survival (interactive prediction profile, Appendix II). The fact that P uptake is limiting on both multiplication ratio and survival in the greenhouse, even at MS levels, suggests that this may be the most limiting element in MS formulation. Considering that P was supplied at much higher concentrations than Ca or Mg during this experiment, it is significant that it is depleted along with these other meso-nutrients (as an aside, P is supplied in lower concentrations than Ca and Mg in MS formulation). Depletion does not prove that growth was limited by the meso-nutrients that were not included in the design, since luxuriant uptake of nutrients often occur in vitro (Adelberg et al. 2010) and these elements were not altered by design. These models, illustrate that P, Ca, and Mg could all be limiting factors to plantlet quality and warrant further investigation.

Correlational Analyses

The ability to measure and analyze so many responses creates the need to sort the responses as more or less important. Multivariate correlations showed how both x-factor nutrients (NH_4^+,NO_3^-, $K^{+)}$, and non-x factor nutrients (Ca, Mg, P); along with sugar and media volume, effected growth (dry mass greenhouse, dry mass laboratory), and plantlet quality (multiplication ratio, survival in the greenhouse). We omitted other measured responses that include: micronutrient ion use (Zn, Cu, Fe, S, Na, B, Cl use), fresh mass gain in laboratory, average fresh mass per plant, sucrose concentration, and osmolarity of residual medium due to relatively lesser correlations to multiplication ratio and plant survival in greenhouse. Manganese (Mn) was unique among micro-nutrients in its correlation with plant quality terms, and was included due to these high correlations.

Figure 5 shows a multivariate correlational analysis of 15 (of the 34 responses tested). Each square (labeled by responses) contains a Pearson's coefficient (r) which measures the strength of linear dependence of two variables. The relationships of Y1 vs. Y2, Y2 vs. Y3, etc. is shown as a number ranging from -1 to +1 to show the direction and strength of relationship between the response pairs. Each colored regions shows responses with stronger correlations $0.60 < r < 1$. Creating this large- multifactor design and arranging the responses based solely upon the strength of correlation informs us of significant relationships in our system.

A strong inverse relationship of relative dry mass in vitro and the dry mass gain in the greenhouse is present (red box, labeled A). This suggests that plantlets from the

laboratory brought enough stored carbohydrate to the greenhouse, so growth in the greenhouse during the first 10-days consisted of: water uptake, root and leaf expansion, and respiration of carbon from stored carbohydrates in the plantlet. Turmeric is a geophyte and utilizes carbohydrate reserves stored in rhizome tissues from prior growth to be utilized in subsequent flushes.

There is a strong positive correlation between fresh mass gain in the greenhouse and multiplication in the laboratory (orange box, labeled B). Plants that multiply quickly in the laboratory grow quickly in the greenhouse (vigor). There was a smaller, positive relationship between multiplication and dry mass gain in the greenhouse (vigor was not necessarily photosynthetic carbon fixation). Multiplication in the lab had a strong positive correlation with greenhouse survival (green box, labeled C). Survival in the greenhouse was not well correlated to the amount of mass gain in the greenhouse, suggesting that these two measures of plant quality do not have similar physiologic causes (the negative osmotic potential increased fresh mass gain, but not plantlet survival). Survival in the greenhouse poorly correlated with relative dry mass from the lab. Anecdotal information circulated among propagators has suggested for many years that the greater dry mass/fresh mass was critical for plantlets to properly acclimatize. This data does not support those assertions. Collectively, A, B, C, and D illustrate that the lab plantlets' fresh or dry mass, or relative dry mass does not predispose the plantlet to survive or fail in the greenhouse.

Multiplication and greenhouse survival was highly correlated with non-x factor elements P, Mg, Mn, and Ca uptake (expressed in a percentage of total use) shown in

light blue box (labeled D). The dry and fresh mass terms had much less relationship to plant quality than the amount of meso-nutrients contained in that mass. Increased uptake of the meso-nutrients elements clearly indicated high quality plants. These four nutrients can be discussed as a group since they cluster tightly as a smaller subset of highly correlated ($0.7 < r < 0.91$) ions where uptake was related to plantlet quality. Other meso- and micro-nutrients (Zn, Cu, Fe, S, Na, B, and Cl- data not shown) did not correlate well with any of the responses or indicate other nutrient use patterns.

The uptake of x-factor nutrient ions did not correlate with multiplication or survival. In the box labeled F there is a high correlation between the two inorganic nitrogen sources used, NO_3^- and NH_4^+, but little correlation between N use and plant quality. Also, K^+ use was not as closely correlated with plant quality as the meso-nutrients.

The volume of media used was correlated with the use of Ca and P (gray box, labeled H). The volume of media used, the amount of K^+ used and the amount of sugar used were all positively correlated with multiplication and greenhouse survival and growth, but the correlations were weak, compared with the meso-nutrients in Block D.

Multiplication and greenhouse survival were well correlated with one another, and these two important measures of plantlet quality were preceded by uptake of P, Mg, Mn, and Ca in vitro. Those plants that multiplied quickly in the laboratory also survived well in the greenhouse. Optimizing medium for these nutrients would likely be important since uptake is associated with desirable plant qualities. Macronutrient use (NO_3^- NH_4^+,and K^+) did not correlate well with any of these quality terms.

Conclusions

Prior work on nutrient medium formulation often lacks responses that are measured after transfer to the greenhouse. In this experiment, we observed similar conditions which allowed rapid plant multiplication and the production of plantlets with high rates of survival and growth in the greenhouse. It was apparent that these nutrient ratios (20 mM macronutrients = 10 mM NO_3^- , 5mM NH_4^+, and 5 mM K^+) were most effective with the lowest numbers of plants in a vessel (3), and much less significant with more plants per vessel. Resolving this type of information required a factorial experiment for evaluation of main effects and interactive effects, including amount and proportion. A full-factorial experiment can result in an impractical large number of experimental units (e.g. 324 experimental units for 4 factors at 3 levels each, 1 mixture response factor at 4 levels, prior to replication). Fractional factorial designs pick subsets of the full-factorial to eliminate experimental units but maintain a similar level of precision. The fractional factorial criterion we chose for point selection, called d-optimality, shrunk this full factorial to approximately 17% of the experimental units of the full factorial (55 experimental units including 5 true replicates) while still allowing a model with linear, quadratic, and second order interactive terms (a response surface model that can model a quadratic). The difficulties in visualizing these solutions are an obstacle to their adaptation. Moving across the response surface using x-coordinate in interactive fashion is one way to overcome this obstacle.

The platform we have used to optimize the macronutrients demonstrates that further exploration of the nutritive space is practical, and will improve plant performance. It is also evident that survival of plantlets in the greenhouse was well correlated with multiplication and uptake of nutrients in the laboratory, specifically Mg, Mn, Ca, and P. The use of this low meso- and micronutrient formulation forced depletion scenarios, and showed which non-x elements to include in future designs. The small size of the described experiment, enabled by advanced designs and computational aids, allows us to rapidly improve tissue culture medium and protocols. A practitioner with modest resources can customize medium and protocols to more closely fit the needs of their biological system.

Table 1.1 – Experimental unit assignment for turmeric (*Curcuma longa* L.) macronutrient experiment with 3 macronutrient ions ($NH4^+$, K^+ NO_3^-), media volume, numbers of explants per vessel and initial sucrose concentration (BRIX%).

NH_4^+ (mM)	K^+ (mM)	NO_3^- (mM)	Media Volume ml	Plants/ Vessel	Sucrose (%)
0	10	10	25	3	3.75
0	10	10	25	6	6
0	10	10	25	9	1.5
0	10	10	35	3	6
0	10	10	45	3	1.5
0	10	10	45	9	6
0	30	30	25	3	1.5
0	30	30	25	9	6
0	30	30	35	6	3.75
0	30	30	45	3	6
0	30	30	45	9	1.5
0	50	50	25	3	6
0	50	50	25	9	1.5
0	50	50	45	3	1.5
0	50	50	45	9	6
1.25	8.75	10	25	3	1.5
1.25	8.75	10	45	3	6
1.25	8.75	10	45	9	1.5
2.5	7.5	10	25	3	3.75
2.5	7.5	10	25	6	6
2.5	7.5	10	25	9	1.5
2.5	7.5	10	35	3	1.5
2.5	7.5	10	45	6	1.5
2.5	7.5	10	45	9	6
5	5	10	25	3	3.75
5	5	10	25	3	3.75
5	5	10	25	6	6
5	5	10	25	6	6
5	5	10	25	9	1.5
5	5	10	35	3	6
5	5	10	35	3	6
5	5	10	45	3	1.5
5	5	10	45	9	6
6.25	43.75	50	25	3	1.5

Table 1.1 Continued

6.25	43.75	50	25	3	6
6.25	43.75	50	45	3	1.5
7.5	22.5	30	25	3	1.5
7.5	22.5	30	35	6	3.75
7.5	22.5	30	45	9	1.5
12.5	37.5	50	25	6	1.5
12.5	37.5	50	25	9	6
12.5	37.5	50	35	9	1.5
12.5	37.5	50	45	3	6
12.5	37.5	50	45	9	6
15	15	30	25	3	1.5
15	15	30	25	9	6
15	15	30	35	6	3.75
15	15	30	35	6	3.75
15	15	30	45	3	6
15	15	30	45	9	1.5
25	25	50	25	3	6
25	25	50	25	9	1.5
25	25	50	45	3	1.5
25	25	50	45	3	1.5
25	25	50	45	9	6

MS standard = 33m (volume), 6 buds, 3% sucrose, 40 mM NO_3^-, 20 mM NH_4^+, K^+

Table 1.2 – Sorted term estimates for multiplication ratio across all 4 laboratory cycles. All term estimates, associated errors, t-ratios, and p-values are included, and model fit analysis follows.

Model: Multiplication Ratio Cycles 1-4				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Macronutrients (mM)	-0.011922	0.001491	-8.00	<.0001
NH_4^+:K^+ Ratio	1.2791976	0.217668	5.88	<.0001
Cycle^2	0.2509728	0.04392	5.71	<.0001
Cycle	-0.17706	0.039786	-4.45	<.0001
Plants/Vessel	-0.07812	0.018404	-4.24	<.0001
NH_4^+:K^+ Ratio *Plants/Vessel	-0.234439	0.079902	-2.93	0.0038
Cycle* Macronutrients (mM)	-0.003374	0.001201	-2.81	0.0055
Macronutrients (mM)*Plants/Vessel	0.0011587	0.000508	2.28	0.0238
Macronutrients (mM)*Sucrose Initial (%)	-0.001439	0.000641	-2.25	0.0259
NH_4^+:K^+ Ratio*Macronutrients (mM)	-0.013207	0.006733	-1.96	0.0513
Cycle*Plants/Vessel	0.0276441	0.015027	1.84	0.0674
Cycle*Media volume (ml)	-0.007722	0.004398	-1.76	0.0808
Media volume (ml) ^{2}	-0.002491	0.001457	-1.71	0.0889
Macronutrients (mM) ^{2}	0.0001091	0.000069	1.58	0.1157
Media volume (ml)	0.0069758	0.005241	1.33	0.1848
Media volume (ml)*Sucrose Initial (%)	0.0030394	0.002366	1.28	0.2006
Sucrose Initial (%)	0.0287635	0.022513	1.28	0.2030
Cycle*Sucrose Initial (%)	-0.021856	0.01929	-1.13	0.2587
NH_4^+:K^+ Ratio^2	-1.765019	1.706892	-1.03	0.3025
Cycle*NH_4^+:K^+ Ratio	0.1902697	0.192983	0.99	0.3255
NH_4^+:K^+ Ratio*Sucrose Initial (%)	-0.090913	0.107089	-0.85	0.3970
Plants/Vessel*Sucrose Initial (%)	0.005607	0.007912	0.71	0.4794
Plants/Vessel^2	0.0098186	0.015417	0.64	0.5250
Macronutrients (mM)*Media volume (ml)	-0.000063	0.000144	-0.44	0.6627
Sucrose Initial (%)^2	0.0031525	0.031142	0.10	0.9195
Media volume (ml)*Plants/Vessel	0.0001725	0.001855	0.09	0.9260
NH_4^+:K^+ Ratio*Media volume (ml)	0.0006949	0.024028	0.03	0.9770
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio =**	9.3797	**Prob > F =**	<.0001
Lack of Fit Hypothesis Test	**F Ratio =**	1.099	**Prob > F =**	0.4286
Error Standard Deviation	0.40352			
Rsquare	0.5778			

Table 1.3 - Sorted term estimates for multiplication ratio in laboratory cycle 4. Term estimates (only showing those with p-value > 0.05), associated errors, t-ratios, and p-values are included, and model fit analysis follows.

Model: Multiplication Ratio Cycle 4				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Macronutrients (mM)	-0.015464	0.003388	-4.56	<.0001
NH_4^+:K^+ Ratio^2	-8.87641	3.194196	-2.78	0.0098
Plants/Vessel	-0.082297	0.033672	-2.44	0.0213
NH_4^+:K^+ Ratio*Plants/Vessel	-0.347847	0.143676	-2.42	0.0225
NH_4^+:K^+ Ratio	0.8714825	0.399971	2.18	0.0382
NH_4^+:K^+ Ratio*Media volume (ml)	0.0894081	0.043548	2.05	0.0499
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio =**	4.3572	**Prob > F =**	0.0002
Lack of Fit Hypothesis Test	**F Ratio =**	4.1089	**Prob > F =**	0.0893
Error Standard Deviation	0.30402			
Rsquare	0.7634			

Table 1.4 - Sorted term estimates for survival in the greenhouse. Term estimates (only showing those with p-value > 0.05), associated errors, t-ratios, and p-values are included, and model fit analysis follows.

Model: Survival in the greenhouse				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
NH_4^+:K^+ Ratio*Plants/Vessel	-0.210474	0.062494	-3.37	0.0022
Macronutrients (mM)	-0.004015	0.001289	-3.11	0.0042
NH_4^+:K^+ Ratio	0.505327	0.171421	2.95	0.0064
Macronutrients (mM) ^{2}	-0.000106	0.000051	-2.1	0.0453
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio =**	3.471500	**Prob > F =**	0.0013
Lack of Fit Hypothesis Test	**F Ratio =**	N/A	**Prob > F =**	N/A
Error Standard Deviation	0.0522			
Rsquare	0.7126			

Table 1.5 - Sorted term estimates for fresh mass change in the greenhouse. Term estimates (only showing those with p-value > 0.05), associated errors, t-ratios, and p-values are included, and model fit analysis follows.

Model: Fresh mass change in greenhouse				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Macronutrients (mM)	-0.003066	0.000771	-3.97	0.0006
Sucrose Initial (%)	0.0303828	0.008971	3.39	0.0027
Plants/Vessel	-0.01964	0.00673	-2.92	0.008
NH_4^+:K^+ Ratio	0.1945822	0.083926	2.32	0.0301
NH_4^+:K^+ Ratio^2	-1.40465	0.611844	-2.3	0.0316
NH_4^+:K^+ Ratio*Plants/Vessel	-0.079258	0.036622	-2.16	0.0416
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio =**	4.4385	**Prob > F =**	0.0005
Lack of Fit Hypothesis Test	**F Ratio =**	8.1868	**Prob > F =**	0.0145
Error Standard Deviation	0.0087			
Rsquare	0.8014			

Table 1.6 - Sorted term estimates for phosphorus use (%) in the laboratory. term estimates (only showing those with p-value > 0.05), associated errors, t-ratios, and p-values are included, and model fit analysis follows.

Model: Phosphorus % used in laboratory				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
NH_4^+:K^+ Ratio	0.7636805	0.201151	3.8	0.0007
NH_4^+:K^+ Ratio^2	-5.975907	1.602538	-3.73	0.0008
Macronutrients (mM)	-0.005505	0.001543	-3.57	0.0013
Plants/Vessel	0.0399254	0.016825	2.37	0.0245
Media volume (ml)	-0.010824	0.005076	-2.13	0.0416
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio =**	4.5596	**Prob > F =**	0.0001
Lack of Fit Hypothesis Test	**F Ratio =**	1.6131	**Prob > F =**	0.3141
Error Standard Deviation	0.0778			
Rsquare	0.7587			

Figure 1.1

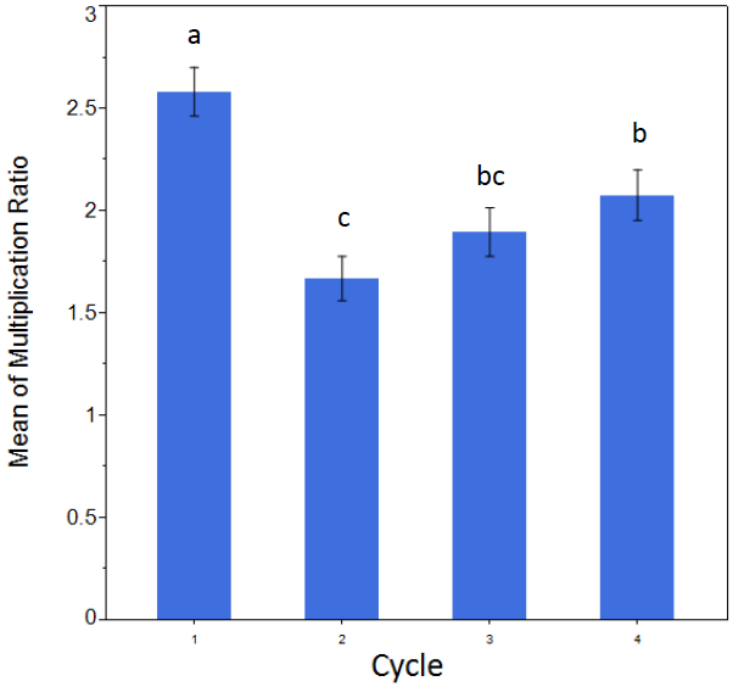
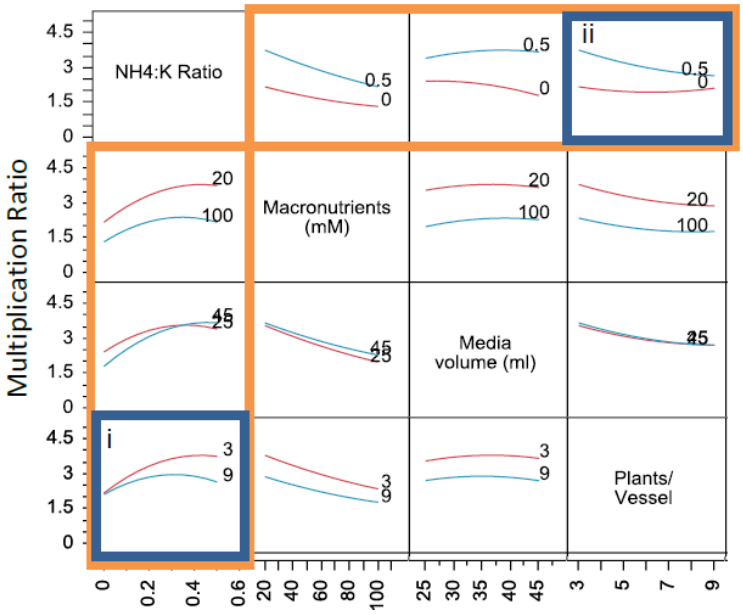
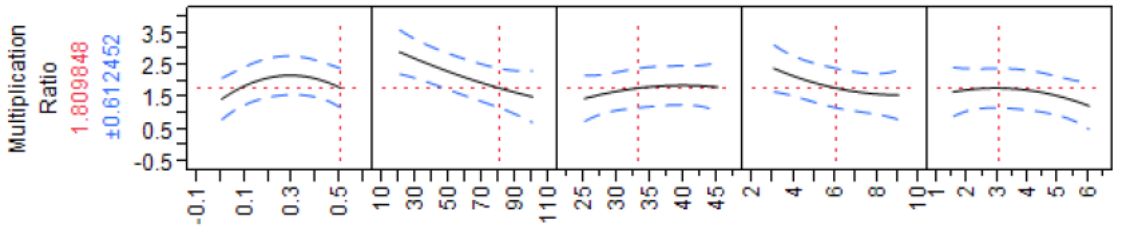


Figure 1.2

A



B



Remembered Settings

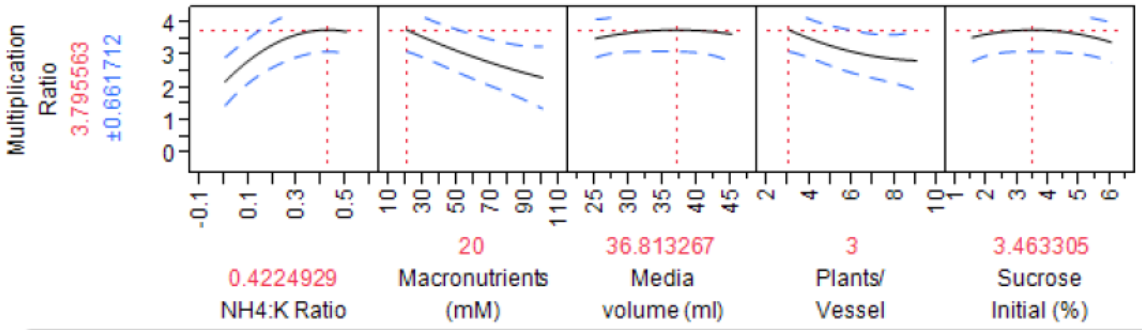
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio
Standard MS	0.5	80	33	6	3	1.8098484
Maximal	0.4224929	20	36.813267	3	3.463305	3.7955625

Differences

Setting A	- Setting B	Multiplication Ratio
Maximal	Standard MS	1.9857141

Figure 1.2 continued

C



Remembered Settings

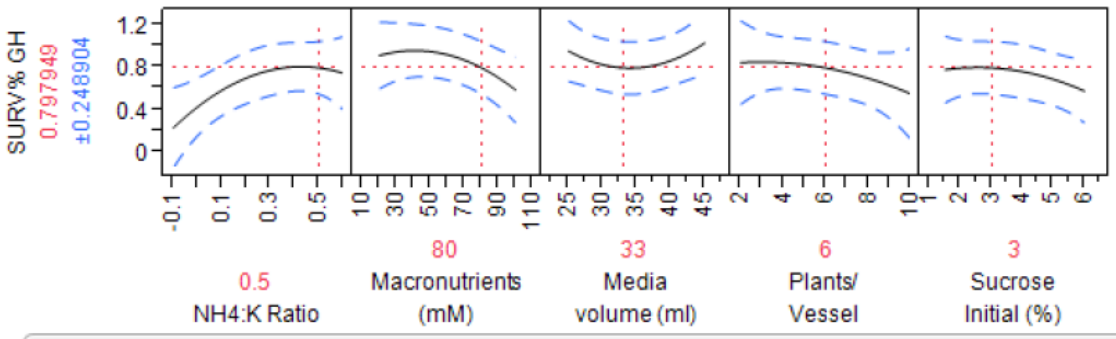
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio
Standard MS	0.5	80	33	6	3	1.8098484
Maximal	0.4224929	20	36.813267	3	3.463305	3.7955625

Differences

Setting A	- Setting B	Multiplication Ratio
Maximal	- Standard MS	1.9857141

Figure 1.3

A



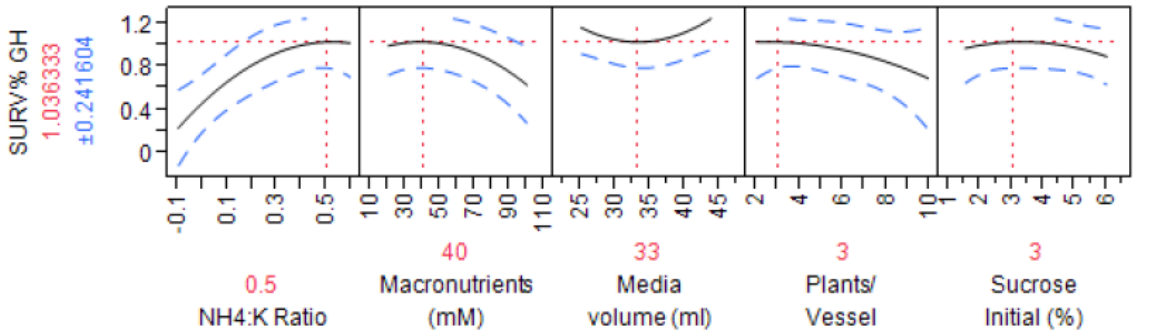
Remembered Settings

Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	SURV% GH
Standard MS	0.5	80	33	6	3	0.7979485
Maximal Survival	0.5	40	33	3	3	1.0363328

Differences

Setting A	- Setting B	SURV% GH
Maximal Survival	Standard MS	0.2383843

B



Remembered Settings

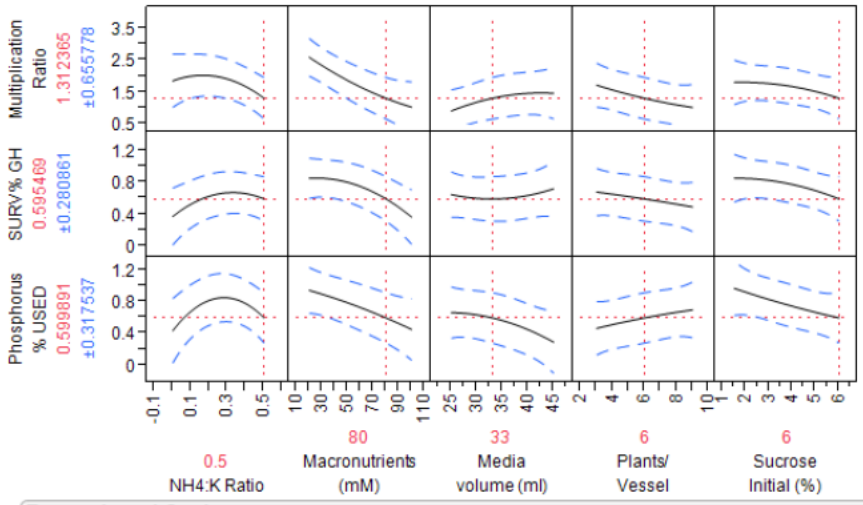
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	SURV% GH
Standard MS	0.5	80	33	6	3	0.7979485
Maximal Survival	0.5	40	33	3	3	1.0363328

Differences

Setting A	- Setting B	SURV% GH
Maximal Survival	Standard MS	0.2383843

Figure 1.4

A



Remembered Settings

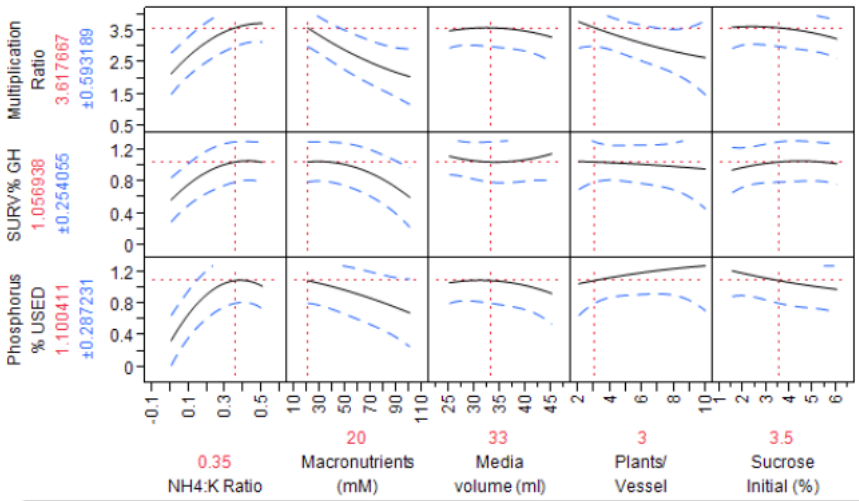
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio	SURV% GH	Phosphorus % USED
Standard MS	0.5	80	33	6	6	1.3123655	0.595469	0.5998915
Compromise Optimal	0.25	20	33	3	3.5	3.3636813	0.9896977	1.0160962

Differences

Setting A	- Setting B	Multiplication Ratio	SURV% GH	Phosphorus % USED
Compromise Optimal	Standard MS	2.0513158	0.3942287	0.4162047

Figure 1.4 continued

B



Remembered Settings

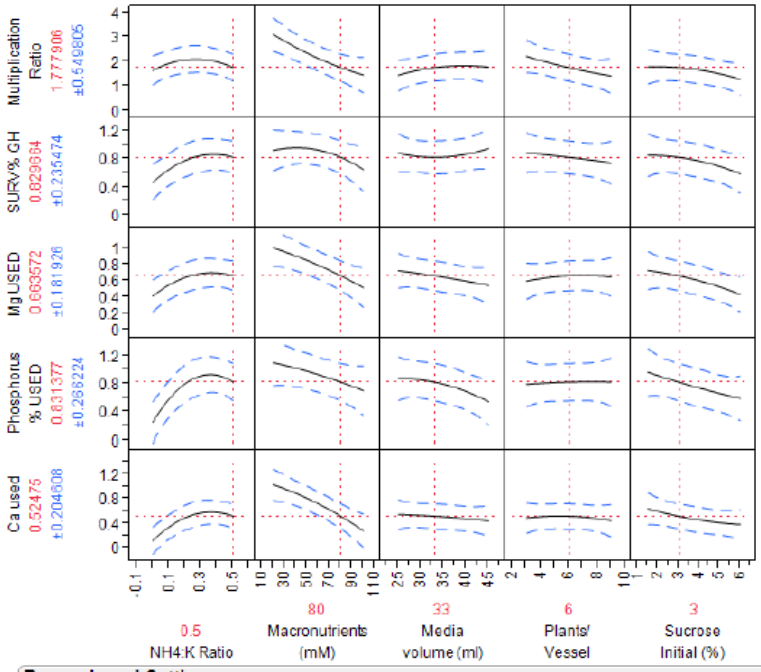
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio	SURV% GH	Phosphorus % USED
Standard MS	0.5	80	33	6	3	1.777906	0.8296643	0.8313767
Compromise Optimal	0.35	20	33	3	3.5	3.6176668	1.0569376	1.1004108

Differences

Setting A	- Setting B	Multiplication Ratio	SURV% GH	Phosphorus % USED
Compromise Optimal	Standard MS	1.8397608	0.2272734	0.2690341

Figure 1.5

A



Remembered Settings

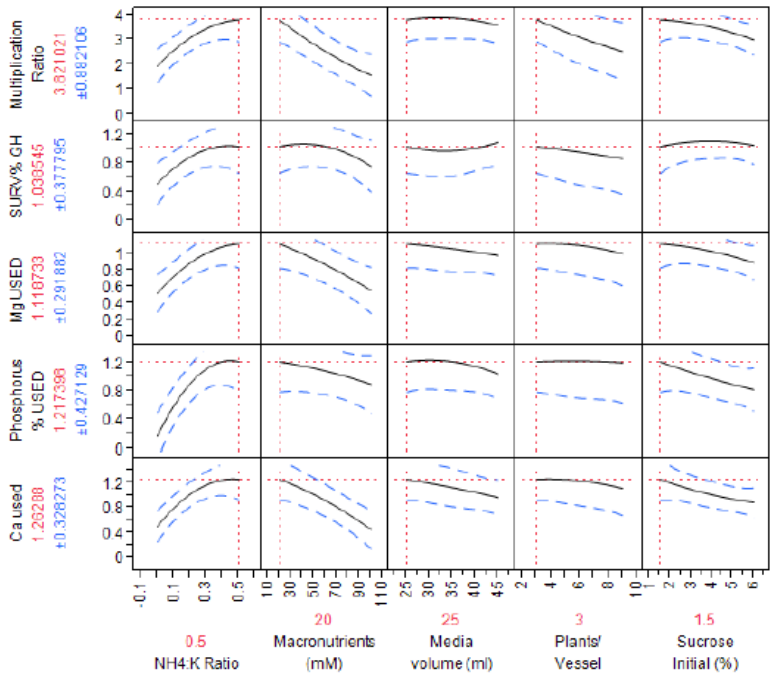
Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio	SURV% GH	Mg USED	Phosphorus % USED	Ca used
Standard MS	0.5	80	33	6	3	1.777906	0.8296643	0.6635722	0.8313767	0.5247501
Maximal	0.5	20	25	3	1.5	3.8210213	1.0365454	1.1187326	1.2173982	1.2626802

Differences

Setting A	- Setting B	Multiplication Ratio	SURV% GH	Mg USED	Phosphorus % USED	Ca used
Maximal	Standard MS	2.0431153	0.2088812	0.4551605	0.3860215	0.7381301

Figure 1.5 continued

B



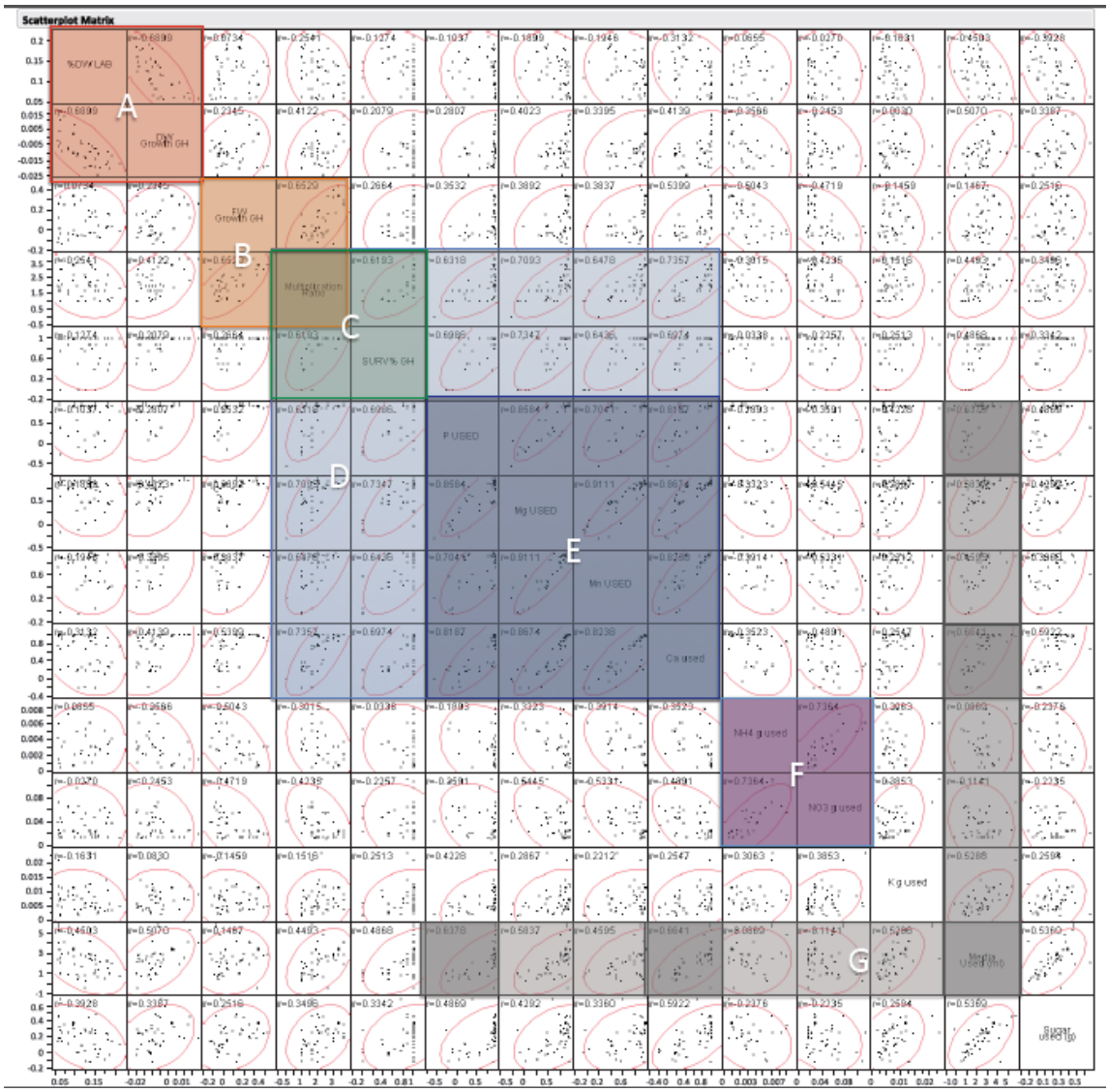
Remembered Settings

Setting	NH4:K Ratio	Macronutrients (mM)	Media volume (ml)	Plants/ Vessel	Sucrose Initial (%)	Multiplication Ratio	SURV% GH	Mg USED	Phosphorus % USED	Ca used
Standard MS	0.5	80	33	6	3	1.777906	0.8296643	0.6635722	0.8313767	0.5247501
Maximal	0.5	20	25	3	1.5	3.8210213	1.0385454	1.1187326	1.2173982	1.2628802

Differences

Setting A	- Setting B	Multiplication Ratio	SURV% GH	Mg USED	Phosphorus % USED	Ca used
Maximal	Standard MS	2.0431153	0.2088812	0.4551605	0.3860215	0.7381301

Figure 1.6


Scatterplot Matrix
%DW LAB
DW Growth GH
FW Growth GH
Multiplication Ratio
SURV% GH
P USED
Mg USED
Mn USED
Ca used
NH4 g used
NO3 g used
Kg used
A
B
C
D
E
F
G

CHAPTER TWO

MACRONUTRIENT OPTIMIZATION FOR MICROPROPAGATION AND ACCLIMATIZATION IN TURMERIC (*Curcuma longa* L.) USING MURASHIGE AND SKOOG MESO- AND MICRONUTRIENT FORMULATION

Abstract

Murashige and Skoog 1962 optimized regeneration of tobacco callus on agar-solidified media, and this media formulation is still widely used for many plant species. However, many MS media formulations may not be optimal for specific plant systems, and such OFAT (one-factor-at-a-time) approaches to media design may not get at true optimal conditions for plant growth. Simultaneous optimization of media components for multiple responses is possible in tissue culture. Successful media formulation requires proper selection of media factors and responses. This macronutrient experiment altered the most massive components in a tissue culture vessel: media volume (25-45 ml), plant density (3-9 divisions), sucrose concentration (1.5-6% m/v), macronutrient concentration (20-100 mM $[NO_3^-]=[NH_4^+]+[K^+]$), and $[NH_4^+]:[K^+]$ ratio (0 to 0.5). Many plantlet quality responses were considered. First, the multiplication stage can improve plantlet quality by increasing quantity; secondly the number of new plants is important where space or labor is the limitation on the process; thirdly the amount of nutrients transferred in the plant from laboratory to greenhouse greatly affects plantlet acclimatization to greenhouse conditions. These three optimization choices were demonstrated to have very different optima as defined. First: multiplication is maximal with low plantlet density (3

plantlets per vessel), high media volume (45 ml), and 4% sucrose in the vessel. Secondly: the number of new plants produced per vessel was highest when the most plants were put in a vessel, at the highest media volume, and highest sucrose % tested. Lastly, those individual plantlets transferred to the greenhouse (100% plantlet survival) which grew the most (via fresh mass gains) were the most massive (fresh mass) came from vessels with: 3 plantlets per vessel, containing no NH_4^+ (all K^+), 45 ml media volume (the highest amounts of media components holding concentrations constant), and the lowest concentration of sucrose tested (1.5%). These three separate optimal illustrate the need for differing tissue culture medium formulations that are propagation scenario dependent. The response surface methodology predicted production of massive plantlets that multiplied quickly in laboratory, and survived the acclimatization phase; while the space clearly identified less than optimal conditions for two of the meso-nutrients (P, Mg) analyzed herein.

Introduction

Murashige and Skoog's revolutionary 1962 media research was a one-factor-at-a-time (OFAT) nutrient experiment that maximized fresh mass of tobacco calli on semi-solid agar medium. Liquid tissue culture produces plants more quickly, with less nutritive-binding; while allowing more stream-lined quantification of nutrients in spent-medium (Adelberg et al. 2010). Quantifying nutrients in liquid in vitro media can be done efficiently and accurately due to lack of water and nutrient binding (Adelberg et al.

2010). By subtraction, nutrient levels in spent medium (thus plant nutritive content) can be compared with field-grown plants in order to determine possible unaccounted deficiencies present in plant tissue culture (Adelberg et al. 2010).

Turmeric (*Curcuma longa* L.) is an important culinary, and medicinal herb native to South and South-East Asia with many secondary-metabolic compounds of interest in various systems of medicine (Shirgurkar et al. 2001). Turmeric cannot be seed propagated because it is a sterile triploid (thus selection is clonal) while varieties are maintained via vegetative propagation (Shirgurkar et al. 2001). Micropropagation enables dissemination of elite materials without the spread of disease. Turmeric was considered here to be a model herbaceous perennial plant for microrhizome multiplication to whole plant tissue culture processes due to the biology of this long-cultivated plant.

Engineering processes utilize optimization modeling, while plant researchers have extended these models to plant propagation processes (Halloran and Adelberg 2011). In plant propagation, the independent variables (x's) that require optimization include (but are not limited to) light, temperature, water, plantlet density, carbon, nitrogen, phosphorus, potassium, etc. The starting point for this experiment was those independent variables that are the most massive components of a tissue culture vessel. These were defined as media volume (25-45 ml), plant density (3-9 divisions), sucrose concentration (1.5-6% m/v), macronutrient concentration (20-100 mM $[NO_3^-]=[NH_4^+]+[K^+]$), and $[NH_4^+]:[K^+]$ ratio (0 to 0.5). Macronutrient concentration and ionic balance were used in order to eliminate ion confounding, according to Niedz and Evens 2006.

The responses plant propagators hope to maximize in micropropagation include dependent variables such as plantlet multiplication, plantlet laboratory dry mass gain, plantlet laboratory fresh mass gain, plantlet greenhouse survival, productivity (minimization of labor or space) with the most new plants produced in a fixed time period, etc. This creates a multi-dimensional optimization space that is difficult to model accurately using least-squares regression. In this experiment we utilized optimality criterion to reduce the experimental space to a useful size for experimental evaluation of said independent variables, while still enabling resolution of main effects, quadratic terms, and second-order interactive effects. Statistical visualization tools now in use enable researchers to explore existing spaces they have created, and in this experiment we sought to create a stream-lined process that resulted in efficient propagation of large, quickly growing plantlets.

We evaluated several measures of plantlet quality: multiplication ratio, the number of new plants produced per vessel, and fresh mass per plantlet. These different measures of quality were evaluated to see if they required very different optimal in vitro conditions. Secondly, greenhouse growth, which had been oft overlooked as a plantlet quality response, was to be evaluated and correlated with laboratory responses in order to determine if laboratory and greenhouse had different optimal conditions. Third, the hypothesis is evaluated whether these in vitro plantlets carried enough media components to the greenhouse from the tissue culture vessel based on published literature about field-grown turmeric plantlets to allow proper acclimatization. These three objectives were

carried out in a fractional-factorial design space, experimental unit assignment using a d-optimality criterion, and modeled via response surface methodology (Table 2.1).

Materials and Methods

Plant Material

Stage I plantlets of *Curcuma longa* L. accession 35-1 were initiated according to methods described by 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. Full-strength commercial bleach (Clorox™, 5.25% sodium-hypochlorite) was used to disinfect shoot tips for 30 s, they were placed on hood surface to dry, and 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, and 30 g sucrose per liter. Medium pH was adjusted to 5.7, and Stage I was carried out in 2 ml of liquid medium in flat-bottomed vials. Stage II plantlets obtained from these cultures were maintained for approximately 5 years by subculture prior to experiments described hereafter.

Immediately prior to experimental conditions, Stage II plantlets were multiplied on similar MS media containing MS vitamin stock, 3 μM benzyl adenine, 3% sucrose. Once adequate stock material was prepared, plants were transferred into 55 different treatment combinations (Table 2.1). The design constrained nitrate concentration (the

predominate anion) to equal the sum of ammonium and potassium (the predominate cations) to reduce pH and counter ion confounding of nutritive effects (Niedz and Evens 2004). All other nutrients were supplied at 100% of MS established concentrations (Murashige and Skoog 1962).

In vitro experiment

Three, 35 day cycles were run where plantlets were sub-cultured and returned to same experimental treatment. During subculture, plants were counted, massed, spent media volume was determined, and sucrose remaining in media was measured as % BRIX on a refractometer (Atago Model N10, Atago Instruments Ltd., Toyko, Japan). Cut buds were transferred into fresh experimental media, and a representative tissue sample was taken for dry mass. Plant material for dry mass was dried for 72 hours at 75°C (relative dry mass= dry/fresh mass). Spent medium was frozen for analysis at the conclusion of cycle 3. Measurements taken were used to calculate media use (initial jar mass – harvested jar mass), sucrose use (initial grams of sugar [brix%*media volume initial]-grams sugar remaining [brix% out in harvested media*media volume remaining]), multiplication ratio (number of plantlets harvested/number of plantlets initial), fresh mass, and dry mass estimate in vitro (relative dry mass per vessel*fresh mass per vessel).

Calculated Responses

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

Multiplication ratio $= (\frac{plantlets\ out\ per\ vessel}{plantlets\ in\ per\ vessel})$ is a standard measurement to determine the relative level of multiplication in a fixed time period (35 day cycle).

Number of new plants per vessel = $total\ plantlets\ out - \frac{plants}{vessel}(initial)$ is the measure of the productivity of each vessel in vitro.

Spent medium analysis

Medium residual from cycle 3 *in vitro* was analyzed by Clemson University's Agricultural Chemical Service using a Thermo Jarrell Ash Model 61E Inductively Coupled Plasma (ICP). Nutrient residual was calculated by ICP concentration determination multiplied by volume of spent medium. The quantity taken up by the plant, was calculated by initial amount less nutrient residual (Adelberg 2010). For the dependent ion variables: K^+ and NO_3^-, use was expressed as mass used by the plantlets (100% of NH_4^+ was depleted from medium), and for the independent ions: P, Ca, Mg, Zn, Cu, Mn, Fe, S, Na, B, and Cl, use was expressed as a percentage of total supplied.

The amount of x-factor nutrient ions used by plant: NH_4^+, K^+, and NO_3^-, was expressed as:

NH_4^+, NO_3^-, and K^+ in grams used = (initial grams NH_4^+, NO_3^-, and K^+) X [1- NH_4^+, NO_3^-, and K^+ ppm in spent medium X Media Out (ml)/(NH_4^+, NO_3^-, and K^+ppm initial X Media Initial (ml).

The amount of non-x nutrient ions used by plant: P, Ca, Mg, Zn, Cu, Mn, Fe, S, Na, B, and Cl, was expressed as a percentage of total supplied:

$$P, Mg, Mn, or\ Ca\ \%\ \text{used} = \left[1 - \frac{P,Mg,Mn,Ca\ ppm\ in\ spent\ medium \times Media\ Out\ (ml)}{P,Mg,Mn,Ca\ ppm\ initial \times Media\ Initial\ (ml)}\right].$$

The preceding formulas were used to determine the ppm (mg/kg) of those nutrients using the following formula, with *n* being the nutrient evaluated.

$$n\ ppm\ in\ dry\ mass\ plant\ tissue\ \left(\frac{mg}{kg}\right) = \left(\frac{n\ grams\ used * 1000}{\frac{Estimated\ dry\ mass}{1000}}\right)$$

Greenhouse experiment

The third cycle was the terminal cycle in vitro and the majority of the tissue went into the greenhouse for acclimatization (and a representative plant went for dry mass). Plantlets were taken from vessels, massed, and rinsed before being placed in individual cells on greenhouse mist bed in Fafard 2-B (minus starter fertilizer) custom soilless mix (Canadian sphagnum peat moss, 3/8" Processed pine bark, perlite, vermiculite, wetting agent, and dolomitic limestone; Fafard Co., Anderson, SC). After 14 days in greenhouse

conditions (Latitude = 34.67350, Longitude = -82.83261; 60% Shade cloth; 6 second on, every 16 min mist cycle during daylight hours) plants were harvested, roots were washed clean of soil, massed again and dried. The difference between mass going into the greenhouse and mass after 14 days in the mist bed was recorded as change. Survival of plantlets was reported as percentage of plantlets per vessel that survived greenhouse acclimatization.

Survival in the greenhouse $= \frac{plantlets\ day\ 14}{plantlets\ day\ 0}$, calculated as plants in greenhouse on day 14 (per vessel) divided by plantlets brought to greenhouse at day 0 (per vessel).

Fresh mass change in the greenhouse

$= fresh\ mass\ day\ 14\ - fresh\ mass\ day\ 0$

Dry mass change in the greenhouse

$= dry\ mass\ day\ 14 - dry\ mass\ day\ 0$

Statistical design of the experiment

This experiment was a fractional factorial of four factors at three levels per factor, with one mixture response factor at 4 levels. The factors altered here were: media volume (25, 35, 45 ml per vessel), plantlet density (3, 6, 9 plantlets per vessel), sucrose concentration (1.5, 3.75, 6 %BRIX), NO_3^- ion concentration (10, 30, 50 mM), and $NH4^+$:K^+ ratio (0:1,

0.5:0.5, 0.25:0.75. 0.125:0.875) with total mM of cations equivalent to NO_3^-. Factor combinations were assigned to 55 experimental units (*in vitro* vessels) according to a d-optimality criterion, with 3 true replicates, as shown in Table 2.1. The form of the model proposed to relate factors to responses was a response surface model. Design, analysis, and graphical visualizations were performed by JMP 9.0 (SAS Inst. Cary, NC).

Results and Discussion

Multiplication ratio across 3, 35-day culture cycles

Multiplication ratio is one measure of laboratory plantlet quality, such that increasing ratios mean faster plantlet production if plantlets are the limitation. Multiplication ratio was well modeled by the independent factors altered in this experiment: media volume, cycle, and plantlet density altered in this experiment, as shown in table 2.2. Table 2.2 shows an *estimate* for terms included in the model for multiplication ratio across cycles, the *standard error* associated with the estimate provided, a *t ratio* (estimate/standard error), and the p-value associated with this estimate which gives the probability that this term has an effect on the model. In Table 2.2 only significant terms (p-value > 0.05) in the response surface model are shown. From the t-ratio's in Table 2.2: the main effects are increased media volume increased multiplication ratio, cycle time decreased multiplication, and increased plants per vessel decreased multiplication.

The quadratic term, Sucrose Initial $(\%)^2$, evaluates a second order effect of initial sucrose which indicates that the effect of sucrose is not a straight line in the response surface. This quadratic term shows a maximal response being in the range tested, around 4% sucrose. Figure 2.1 shows second order interactions, and where initial sucrose in the vessel was too low (1.5%) or too high (6%), regardless of media volume or plants per vessel, plants multiplied slowly. The interaction of Sucrose Initial % with Plants/Vessel showed maximal multiplication at 3 plants per vessel, and the greatest media volume (45 mL), as shown in blue blocks of Figure 2.1. When there were 9 plants in the vessel, the response of sucrose had a lesser effect on multiplication around 4% sucrose, and thus pulled the whole response surface downward. The interaction of Sucrose Initial % with Media volume showed that multiplication was maximized at 45 mL of media, and at the higher sucrose percentage according to the quadratic relationship of sucrose. Each interactive effect can be visualized using this format. However, just visualizing pairs of interactive x-factors misses the power of optimizing responses with multiple variables in a single design (5 factors in this design plus cycle). This model allows maximization of multiple factors in plant tissue culture, however the visualization of these model effects and responses is difficult in 2 dimensions (as in Figure 2.1).

Figure 2.2a shows that multiplication ratio at standard conditions for this vessel size (cycle 3, balanced NH_4^+:K^+ ratio, 80 mmol Macronutrients, 33 ml media volume, 6 plants per vessel, and 3% sucrose) is approximately 4x (or 6 plants in = 24 plants out). The maximal multiplication ratio (Fig. 2.2b) in cycle 3 (as this was the cycle to go to greenhouse) was achieved at the highest media volume (45 ml), raising sucrose level to

4%, and reducing 3 plants per vessel was 5x (or 3 plants in= 15 plants out). Maximal multiplication ratio is important to increase the numbers of a new plant, however for producing the greatest numbers of plantlets in a facility (as in a commercial application), there is a different set of maximal conditions. As labor and space are the most valuable parts of tissue culture, optimization must be done in the number of new plants response dimension to account for manual transfer of plantlets and the space those vessels consume.

Number of new plants in Cycle 3

The number of new plants per vessel is the plantlet quality that gets more at efficiency of the tissue culture vessel. There were different optimal nutrient combinations than shown for multiplication ratio. Table 2.3 shows the model fit of number of new plants produced per vessel in cycle 3 (those parameters with p-value > 0.05 were omitted). Plants per vessel initiated is a significant term in the number of new plants produced per vessel, and more plants in (plants per vessel) creates more plants out (number of new plants). The media volume by sucrose interactive effect and media volume by macronutrient effect shows as more plants were added to a vessel, more resources were needed. More media means more sugar based on volume at a given percentage, and more media means more macronutrient based on volume at a given concentration. Thus, these two interactive terms influence the amount of sucrose and salt respectively. At the low media volume (25 ml per vessel), sucrose % has very little effect

on the number of new plants. This leads to the question that some other media ingredient, other than those tested was limiting.

Figure 2.3a and 2.3b show that by increasing plants per vessel from 6 buds to 9, increasing media volume from 33ml to 45ml, and increasing sucrose from 3% to almost 6%; number of new plants can be increased approximately 1.5 times (17 to 26). Also, we can see in Figure 2.3b that at the low media volume, macronutrient concentration had very little effect on the number of new plants generated; while at the high media volume we can increase the number of plantlets produced per vessel from 17 to 26 plants by increasing concentration of macronutrients. The next question with this effect of media volume would be was water the limiting factor, however we observed no vessels which were depleted of media and only one vessel of 55 had less than 10 ml remaining at the conclusion of the third cycle. So, if water, macronutrients, and sucrose are not limiting, there is another yet overlooked aspect of the media volume that is.

Average fresh mass per plant

Average fresh mass per plant is a different measure of plantlet quality, and we found that it's optimal values confer plantlet quality in yet another dimension. Table 2.4 shows all factors studied were important main effects in the model of average fresh mass per plantlet.

Higher concentration of macronutrients, and more media volume both increase average fresh mass per plantlet; while plants per vessel and sucrose concentration initial

reduce the fresh mass per plantlet. NH_4^+:K^+ ratio decreases fresh mass per plantlet in the laboratory, which suggested elimination of the ammonium ion in favor of potassium in order to produce massive plantlets. The interaction of media volume by sucrose concentration indicated that initial sucrose concentration was most important at the higher media volumes, but as the main effect suggest more sucrose was not better to produce massive plantlets.

Figure 2.4a and 2.4b show that by removing ammonium, increasing macronutrient concentration, increasing media volume (or amount of salt and sugar at fixed concentrations), reducing the number of plantlets, and reducing sucrose produced massive plantlets. Those few (3 buds per vessel) plants in those vessels received the most potassium (no ammonium) and nitrate which illustrates the need for a metabolic shift (reduce sugar) from plants in the laboratory which are photomixotrophic or heterotrophic to the photoautotrophic greenhouse conditions.

Evaluation of greenhouse growth

Rearing more plants per vessel at higher plant density may have benefit in micropropagation if those plants are competent to grow in the greenhouse. All of the 144 total plantlets transferred from cycle 3 in laboratory acclimatized to greenhouse conditions. Greenhouse quality was evaluated by measuring fresh mass during 14 days of acclimatization. The model for fresh mass gain in the greenhouse (Table 2.5) showed that the ratio of macronutrients and the number of plants per vessel decreased fresh mass gain

in the greenhouse. Media volume increased fresh mass gain in the greenhouse, and the initial concentration of sucrose interacted with both media volume and macronutrient concentration.

Figure 2.5a shows a positive fresh mass change (0.32 grams) in the greenhouse at the standard conditions for this vessel size. Acclimatization is a lag in growth while plantlets adjust from photomixotrophic or heterotrophic conditions to photoautotrophic metabolic conditions in the greenhouse. Figure 2.5b shows us that by replacing NH_4^+ with K+, decreasing macronutrients slightly, increasing media volume, decreasing plants per vessel, and increasing sucrose we can increase greenhouse fresh mass gain (from 0.32 to 1.13 grams).

The fresh mass gains in greenhouse model shows that replacing ammonium with potassium would benefit plant quality regarding greenhouse growth. Ammonium can possibly be completely removed from media prior to acclimatization. Macronutrients do not appear to be limiting in the greenhouse growth, and it is again obvious that those plants who had the most access to media volume grew the best in the greenhouse even though water was not the limiting factor. In apparent contradiction to the average fresh mass per plantlet model, it is apparent that more sucrose produces greater initial fresh mass gain in the greenhouse; however this is due to sucrose as an energy term in photosynthesis and whole plant translocation of sugars.

The plants with the best growth in the greenhouse were the largest plants from the lab. They came from vessels with the fewest numbers of plants in with the most media volume. They did use the greater amounts of macronutrients than plants with lesser

growth potential. It is possible that the availability of meso- and micronutrients, not in the experimental design were limiting growth in the greenhouse. Thus, we compared the plants internal nutrient concentration, determined by spent medium analysis, with other published analyses of plant mineral nutrition in healthy, productive fields of turmeric.

Nutrient comparisons

As more plants were produced per vessel, the concentrations of these elements decreased. The tissue culture vessel is a closed system, and logically if more plantlets are being produced they would each have lowered amounts of nutrient available to them. It is possible to see whether the availability of nutrients not in this study were likely limiting growth in those vessel which were producing the most plants. Phosphorus has been shown to be rapidly depleted by turmeric grown in MS medium with 2x phosphate (Adelberg 2010).

It is evident from figures 2.6a and 2.6b that this design allowed a possible increase dry mass of P in plantlets from MS levels (compare b to a). Also, figures 2.7a and 2.7b show the ability of this design space to increase dry mass of Mg in plantlets when compared with standard MS 1962 levels.

Kumar et al. 2003 (as presented in Table 2.6) shows nutrient ranges converted for field-grown turmeric that were compared with the elemental concentrations estimated for these experimental plantlets. Figures 2.8, 2.9, 2.10, and 2.11 plot the ppm concentration in dry mass of tissue (y-axis) of the elements in each vessel, against the number of new

plants per vessel (x-axis). These figures show the published data as in table 2.6 (in dotted lines) for all elements that were relevant to this study. P and Mg were included due to possible deficiency; while Ca is shown for sufficiency, and K was included in the experimental design.

Figure 2.8 shows concentration of P in dry mass of plant tissue from the experiment with new plants produced per vessel to create spread, and also illustrate the need for more of all of these meso-nutrients if the goal is more plantlets. Figure 2.8 represents that plantlets from the majority of vessels (31 out of 55) fall below the sufficient range for P marked by the upper and lower dotted lines, while only 24 are considered in the sufficient range according to published data on field-grown plants of turmeric. All eight vessels that produced more than 20 new plants had inadequate P nutrition when compared to the field grown tissue standards of Kumar et al. (2003).

Figure 2.9 shows how Ca along with appeared to be sufficient for turmeric in the culture conditions tested (S, Fe, Mn, B, Cu, Zn, Mo, Na, Al also showed sufficiency: data not shown for brevity's sake). Figure 2.8 highlights that Mg was possibly deficient in 51 out of 55 vessels. This elemental analysis illustrates the need for improving the tissue culture environment for at least P, and Mg. K, being an independent variable, cannot be properly evaluated as it is confounded in the experimental design, and Figure 2.9 shows a different spread across plants per vessel that is illogical because of its confounding with plants per vessel in the experimental design.

Conclusions

This experimental procedure optimized numerous responses useful in evaluation of tissue culture processes. As stated, selection of proper x's and their desired interactions cannot be overlooked for this large subset of plant biotechnology; however a problem (identified here) in data dense experimentation is not only selection of proper response variables to optimize these complex systems, but also proper modeling approaches, experimental statistical model development, and visualization of aforementioned models. Plantlet quality was defined in three very distinct ways, depending upon the desired result. First, the multiplication stage was improved plantlet quality by increasing quantity; secondly the number of new plants was important where space or labor was the limitation on the process; thirdly the amount of nutrients transferred in the plant from laboratory to greenhouse greatly affected plantlet acclimatization to greenhouse conditions. These three optimization choices were demonstrated to have very different optima as defined in this experiment. First: multiplication is maximal with low plantlet density (3 plantlets per vessel), high media volume (45 ml), and 4% sucrose in the vessel. Secondly: the number of new plants produced per vessel was highest when the most plants were put in a vessel, at the highest media volume, and highest sucrose concentration tested. Lastly, those individual plantlets transferred to the greenhouse (100% plantlet survival) which grew the most (via fresh mass gains) were the most massive (fresh mass) came from vessels with: 3 plantlets per vessel, containing no NH_4^+ (all K^+), 45 ml media volume (the highest amounts of media components holding concentrations constant), and the lowest

concentration of sucrose tested (1.5%). These three separate optima illustrate the need for differing tissue culture medium formulations that are propagation scenario dependent. This experiment, at full MS meso- and micronutrient values, identified P and Mg may be deficient in standard formulations when compared with field-grown plantlets of turmeric.

Table 2.1 – Experimental unit assignment for turmeric (*Curcuma longa* L.) macronutrient experiment with 3 macronutrient ions ($NH4^+$, K^+ NO_3^-), media volume, numbers of explants per vessel and initial sucrose concentration (BRIX%).

NH_4^+ (mM)	K^+ (mM)	NO_3^- (mM)	Media Volume ml	Plants/ Vessel	Sucrose (%)
0	10	10	25	3	3.75
0	10	10	25	6	6
0	10	10	25	9	1.5
0	10	10	35	3	6
0	10	10	45	3	1.5
0	10	10	45	9	6
0	30	30	25	3	1.5
0	30	30	25	9	6
0	30	30	35	6	3.75
0	30	30	45	3	6
0	30	30	45	9	1.5
0	50	50	25	3	6
0	50	50	25	9	1.5
0	50	50	45	3	1.5
0	50	50	45	9	6
1.25	8.75	10	25	3	1.5
1.25	8.75	10	45	3	6
1.25	8.75	10	45	9	1.5
2.5	7.5	10	25	3	3.75
2.5	7.5	10	25	6	6
2.5	7.5	10	25	9	1.5
2.5	7.5	10	35	3	1.5
2.5	7.5	10	45	6	1.5
2.5	7.5	10	45	9	6
5	5	10	25	3	3.75
5	5	10	25	3	3.75
5	5	10	25	6	6
5	5	10	25	6	6
5	5	10	25	9	1.5
5	5	10	35	3	6
5	5	10	35	3	6
5	5	10	45	3	1.5
5	5	10	45	9	6
6.25	43.75	50	25	3	1.5

Table 2.1 Continued

6.25	43.75	50	25	3	6
6.25	43.75	50	45	3	1.5
7.5	22.5	30	25	3	1.5
7.5	22.5	30	35	6	3.75
7.5	22.5	30	45	9	1.5
12.5	37.5	50	25	6	1.5
12.5	37.5	50	25	9	6
12.5	37.5	50	35	9	1.5
12.5	37.5	50	45	3	6
12.5	37.5	50	45	9	6
15	15	30	25	3	1.5
15	15	30	25	9	6
15	15	30	35	6	3.75
15	15	30	35	6	3.75
15	15	30	45	3	6
15	15	30	45	9	1.5
25	25	50	25	3	6
25	25	50	25	9	1.5
25	25	50	45	3	1.5
25	25	50	45	3	1.5
25	25	50	45	9	6

MS standard = 33m (volume), 6 buds, 3% sucrose, 40 mM NO_3^-, 20 mM NH_4^+, K^+

Table 2.2 – Sorted term estimates for multiplication ratio of in vitro turmeric plantlets following 4th 35-day culture cycle under treatments conditions (p-values shown < alpha=0.05)

Model: Multiplication ratio over 3 cycles in vitro				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Media Volume	0.0473	0.0090	5.24	<.0001
Cycle	-0.4616	0.0930	-4.97	<.0001
Plants/Vessel	-0.1426	0.0330	-4.32	<.0001
Sucrose Initial %^2	-0.1868	0.0551	-3.39	0.0009
Media Volume*Sucrose Initial %	0.0102	0.0041	2.48	0.0143
Media Volume*Plants/Vessel	-0.0065	0.0032	-1.99	0.0482
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio=**	4.3730	**Prob>F=**	<.0001
Lack of Fit Hypothesis Test	**F Ratio=**	0.5099	**Prob>F=**	0.9766
Error Standard Deviation	0.9570			
RSquare	0.4629			

Table 2.3 Sorted term estimates for number of new turmeric plantlets following the 4th 35-day culture cycle under treatments conditions, only showing parameters with p-values less than 0.05.

Model: Number of new plants per vessel in laboratory cycle 3				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Plants/Vessel	1.6338422	0.258815	6.31	<.0001
Media Volume*Sucrose Initial (%)	0.0828652	0.032169	2.58	0.0145
Macronutrients*Media Volume	0.0042046	0.001965	2.14	0.0397
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio=**	3.7871	**Prob>F=**	0.0003
Lack of Fit Hypothesis Test	**F Ratio=**	2.4832	**Prob>F=**	0.1571
Error Standard Deviation	19.4796			
Rsquare	0.6902			

Table 2.4 – Sorted term estimates for average fresh mass per plantlet in laboratory following the4th 35-day culture cycle under treatments conditions, only showing parameters with p-values less than 0.05.

Model: Average fresh mass per plantlet in laboratory				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Macronutrients	0.004226	0.000943	4.48	<.0001
Media Volume	0.013782	0.003438	4.01	0.0003
Plants/Vessel	-0.04786	0.012569	-3.81	0.0006
Sucrose Initial (%)	-0.03766	0.015139	-2.49	0.0179
NH4:K Ratio	-0.29705	0.145381	-2.04	0.0488
Media Volume*Sucrose Initial (%)	0.003174	0.001562	2.03	0.05
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio=**	4.7077	**Prob>F=**	<.0001
Lack of Fit Hypothesis Test	**F Ratio=**	17.5668	**Prob>F=**	0.0023
Error Standard Deviation	0.0459			
Rsquare	0.7347			

Table 2.5 Sorted term estimates for fresh mass gain of turmeric plantlets in the greenhouse following the 4th 35-day culture cycle under treatment conditions, only showing parameters with p-values less than 0.05.

Model Fresh mass gain in the greenhouse				
Components of Best Fit Model				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
NH4:K Ratio	-0.58883	0.184212	-3.2	0.003
Media Volume	0.01377	0.004357	3.16	0.0033
Plants/Vessel	-0.04568	0.015926	-2.87	0.007
Macronutrients*Sucrose Initial (%)	0.001177	0.000534	2.21	0.0342
Media Volume*Sucrose Initial (%)	0.004235	0.00198	2.14	0.0397
Model Fit Analysis				
Whole Model Hypothesis Test	**F Ratio=**	2.7968	**Prob>F=**	0.004
Lack of Fit Hypothesis Test	**F Ratio=**	2.071	**Prob>F=**	0.2139
Error Standard Deviation	0.0737			
Rsquare	0.6219			

Table 2.6 – Converted nutrient values from Kumar et al. 2003 published nutrient data

Element	*Kumar et al. 2003 mg/kg*
N	7200 - 36500
P	2500 - 15600
K	20900 - 81300
Ca	1200 - 8500
Mg	3700 - 10000
S	900 - 7500
Fe	210 - 1825
Mn	14 - 118
B	5.28 - 30.7
Cu	16.2 - 59.4
Zn	43.2 - 96.1
Mo	ND
Na	900 - 3900
Al	ND

ND = no data

Figure 2.1

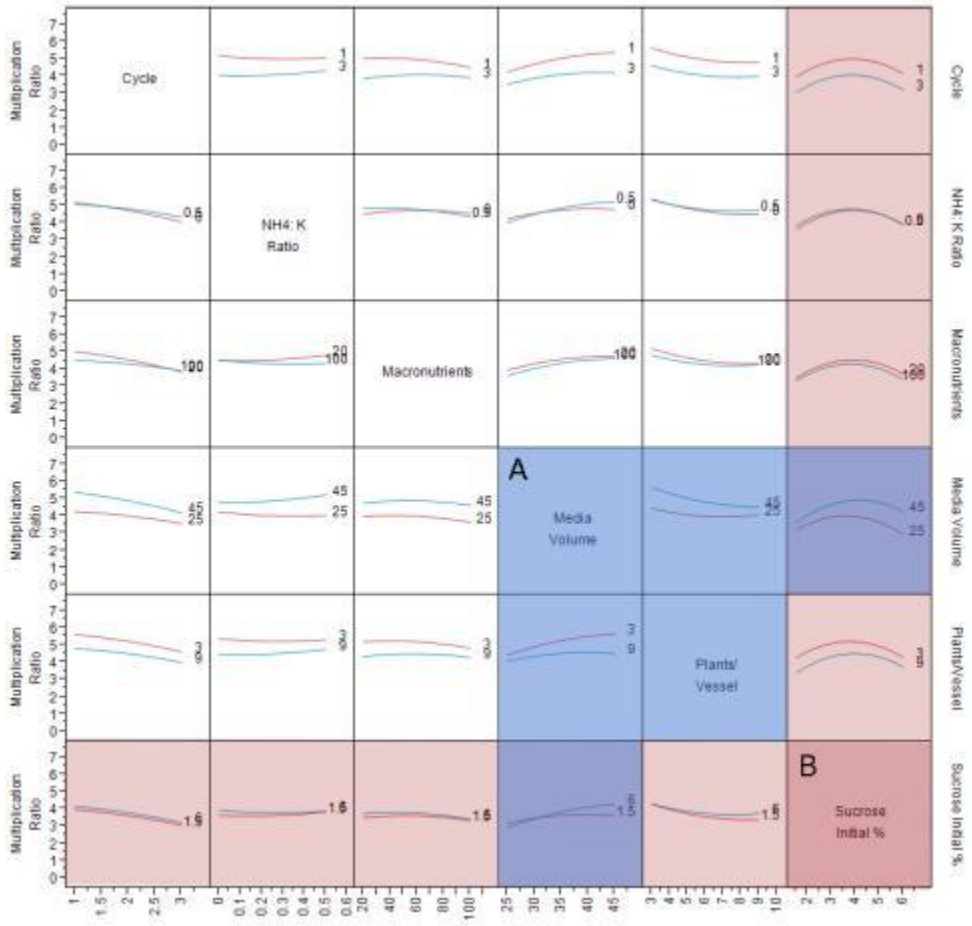
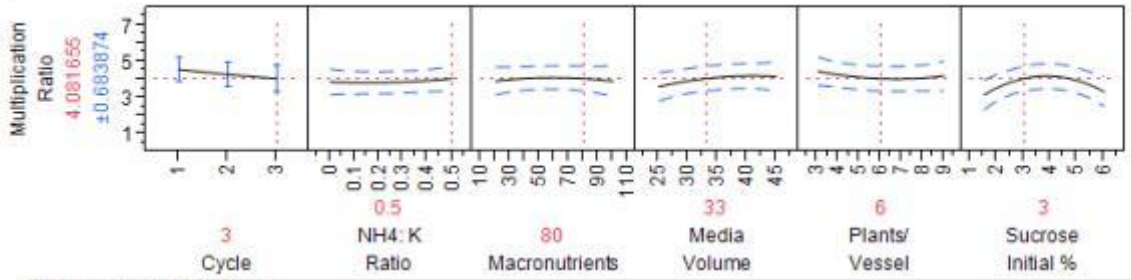

Multiplication Ratio
Cycle
NH4: K Ratio
Macronutrients
A
Media Volume
Plants/ Vessel
B
Sucrose Initial %
Cycle
NH4: K Ratio
Macronutrients
Media Volume
Plants/Vessel
Sucrose Initial %

Figure 2.2

Figure 2a



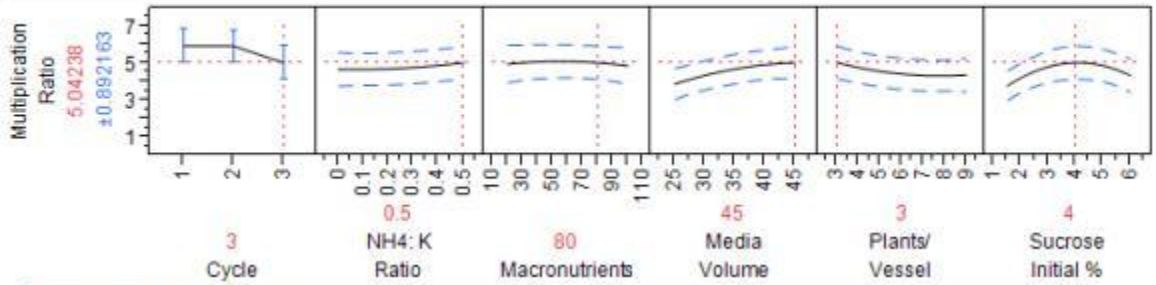
Remembered Settings

Setting	Cycle	NH4: K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial %	Multiplication Ratio
Cycle 3 MS Standard	3	0.5	80	33	6	3	4.0816548
Cycle 3 Maximization	3	0.5	80	45	3	4	5.0423799

Differences

Setting A	- Setting B	Multiplication Ratio	Desirability
Cycle 3 Maximization	Cycle 3 MS Standard	0.9607251	

Figure 2b



Remembered Settings

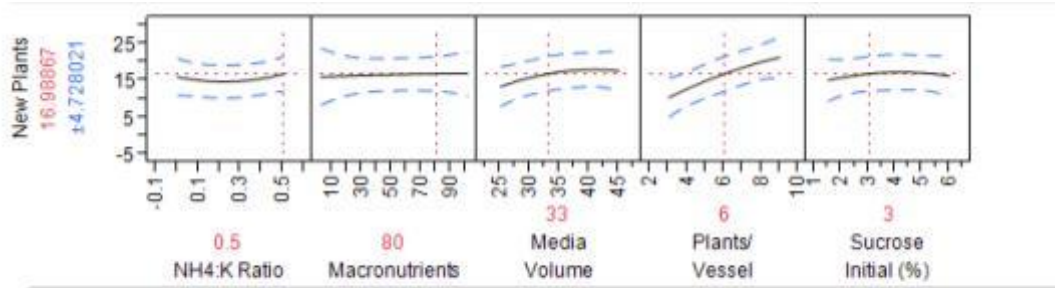
Setting	Cycle	NH4: K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial %	Multiplication Ratio
Cycle 3 MS Standard	3	0.5	80	33	6	3	4.0816548
Cycle 3 Maximization	3	0.5	80	45	3	4	5.0423799

Differences

Setting A	- Setting B	Multiplication Ratio
Cycle 3 Maximization	Cycle 3 MS Standard	0.9607251

Figure 2.3

A



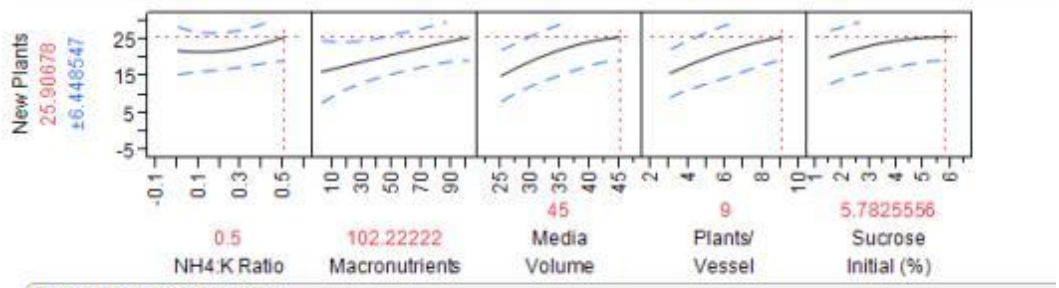
Remembered Settings

Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	New Plants
MS Standard	0.5	80	33	6	3	16.98867
Maximal New Plants Out	0.5	102.22222	45	9	5.7825556	25.906779

Differences

Setting A	- Setting B	New Plants	Desirability
Maximal New Plants Out	MS Standard	8.9181098	.

B



Remembered Settings

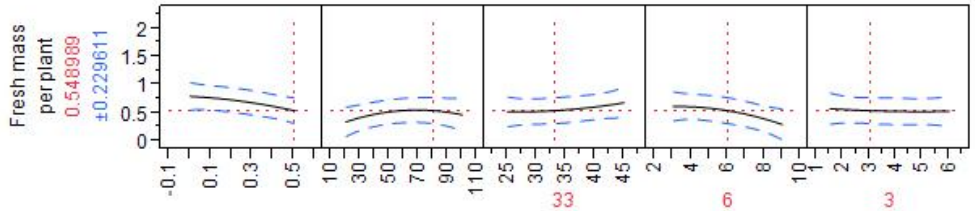
Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	New Plants
MS Standard	0.5	80	33	6	3	16.98867
Maximal New Plants Out	0.5	102.22222	45	9	5.7825556	25.906779

Differences

Setting A	- Setting B	New Plants	Desirability
Maximal New Plants Out	MS Standard	8.9181098	.

Figure 2.4

A



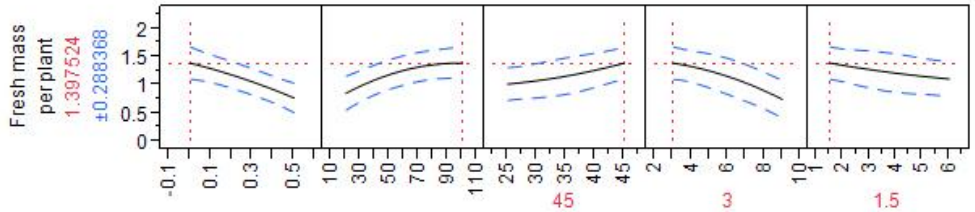
Remembered Settings

Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	Fresh mass per plant
MS Standard	0.5	80	33	6	3	0.5489893
Maximal fresh mass per plantlet	0	100	45	3	1.5	1.3975241

Differences

Setting A	- Setting B	Fresh mass per plant	Desirability
Maximal fresh mass per plantlet	MS Standard	0.8485347	.

B



Remembered Settings

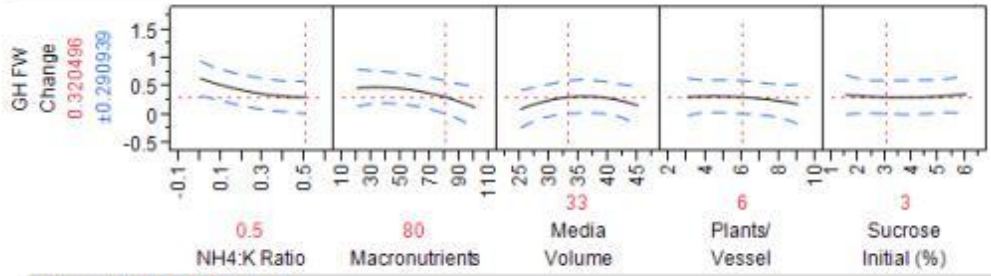
Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	Fresh mass per plant
MS Standard	0.5	80	33	6	3	0.5489893
Maximal fresh mass per plantlet	0	100	45	3	1.5	1.3975241

Differences

Setting A	- Setting B	Fresh mass per plant	Desirability
Maximal fresh mass per plantlet	MS Standard	0.8485347	.

Figure 2.5

5a



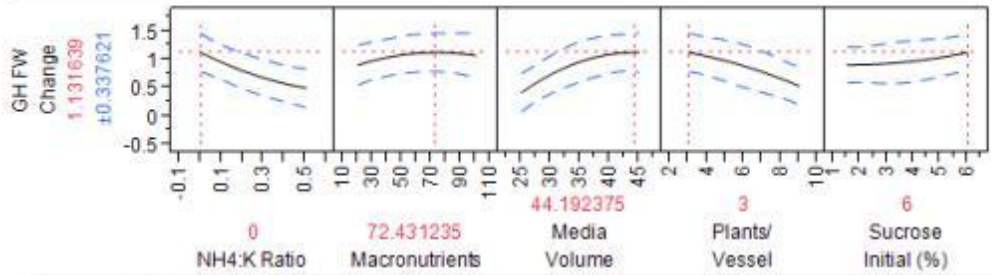
Remembered Settings

Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	GH FW Change
◉MS Standard	0.5	80	33	6	3	0.3204955
○Maximal Fresh Weight Change in Greehouse	0	72.431235	44.192375	3	6	1.1316395

Differences

Setting A	- Setting B	GH FW Change	Desirability
Maximal Fresh Weight Change in Greehouse	MS Standard	0.8111439	

5b



Remembered Settings

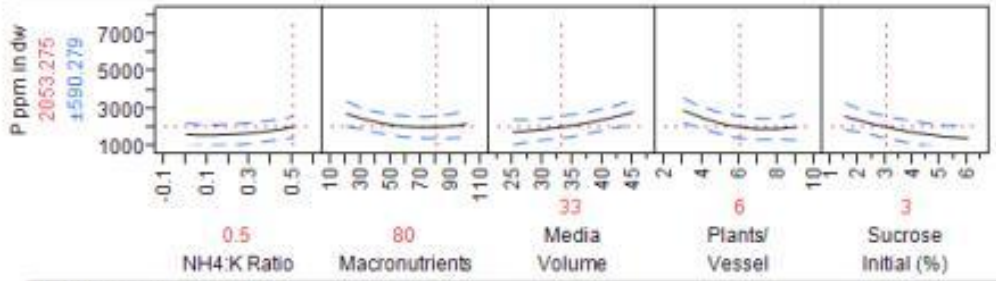
Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	GH FW Change
○MS Standard	0.5	80	33	6	3	0.3204955
◉Maximal Fresh Weight Change in Greehouse	0	72.431235	44.192375	3	6	1.1316395

Differences

Setting A	- Setting B	GH FW Change	Desirability
Maximal Fresh Weight Change in Greehouse	MS Standard	0.8111439	

Figure 2.6

A



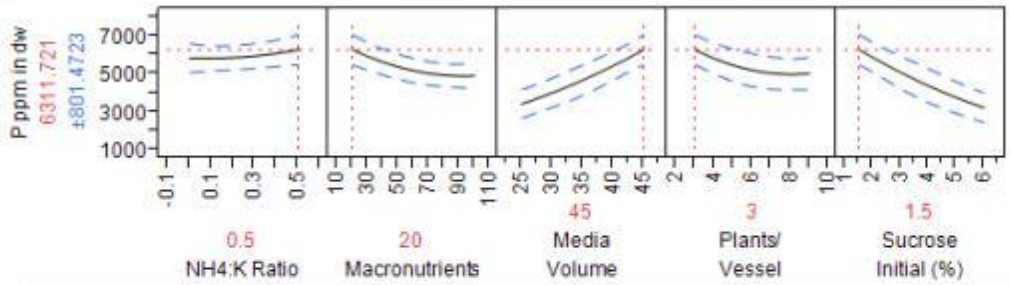
Remembered Settings

Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	P ppm in dw
◉MS Standard	0.5	80	33	6	3	2053.2746
○Optimal P in tissue mass	0.5	20	45	3	1.5	6311.7213

Differences

Setting A	- Setting B	P ppm in dw	Desirability
Optimal P in tissue mass	MS Standard	4258.4467	

B



Remembered Settings

Setting	NH4:K Ratio	Macronutrients	Media Volume	Plants/ Vessel	Sucrose Initial (%)	P ppm in dw
○MS Standard	0.5	80	33	6	3	2053.2746
◉Optimal P in tissue mass	0.5	20	45	3	1.5	6311.7213

Differences

Setting A	- Setting B	P ppm in dw	Desirability
Optimal P in tissue mass	MS Standard	4258.4467	

Figure 2.7

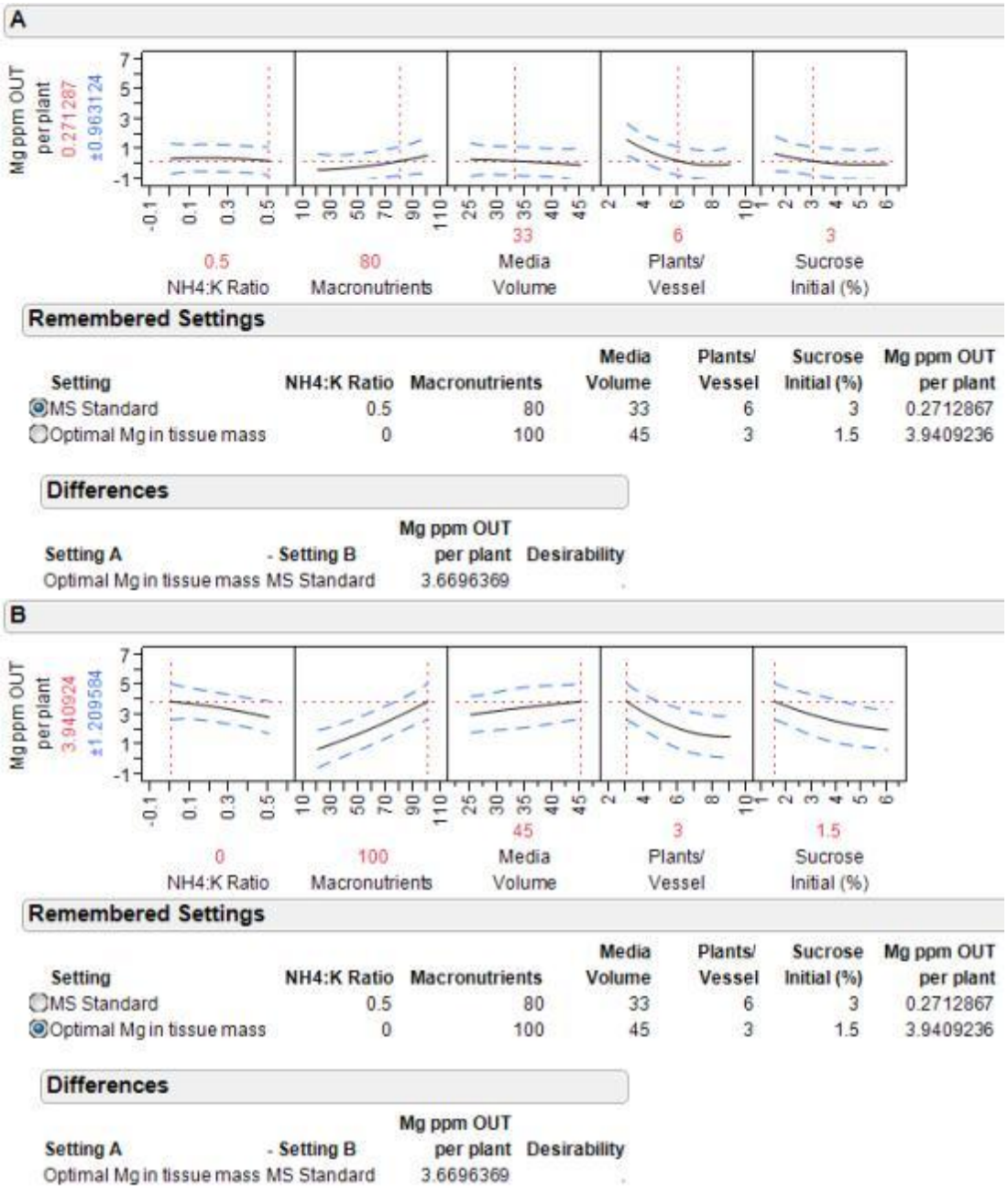

A
Mg ppm OUT per plant
0.271287
±0.963124
0.5
NH4:K Ratio
80
Macronutrients
33
Media Volume
6
Plants/ Vessel
3
Sucrose Initial (%)
Remembered Settings
Setting | NH4:K Ratio | Macronutrients | Media Volume | Plants/ Vessel | Sucrose Initial (%) | Mg ppm OUT per plant
MS Standard | 0.5 | 80 | 33 | 6 | 3 | 0.2712867
Optimal Mg in tissue mass | 0 | 100 | 45 | 3 | 1.5 | 3.9409236
Differences
Setting A - Setting B | Mg ppm OUT per plant | Desirability
Optimal Mg in tissue mass MS Standard | 3.6696369 |
B
Mg ppm OUT per plant
3.940924
±1.209584
0
NH4:K Ratio
100
Macronutrients
45
Media Volume
3
Plants/ Vessel
1.5
Sucrose Initial (%)
Remembered Settings
Setting | NH4:K Ratio | Macronutrients | Media Volume | Plants/ Vessel | Sucrose Initial (%) | Mg ppm OUT per plant
MS Standard | 0.5 | 80 | 33 | 6 | 3 | 0.2712867
Optimal Mg in tissue mass | 0 | 100 | 45 | 3 | 1.5 | 3.9409236
Differences
Setting A - Setting B | Mg ppm OUT per plant | Desirability
Optimal Mg in tissue mass MS Standard | 3.6696369 |

Figure 2.8

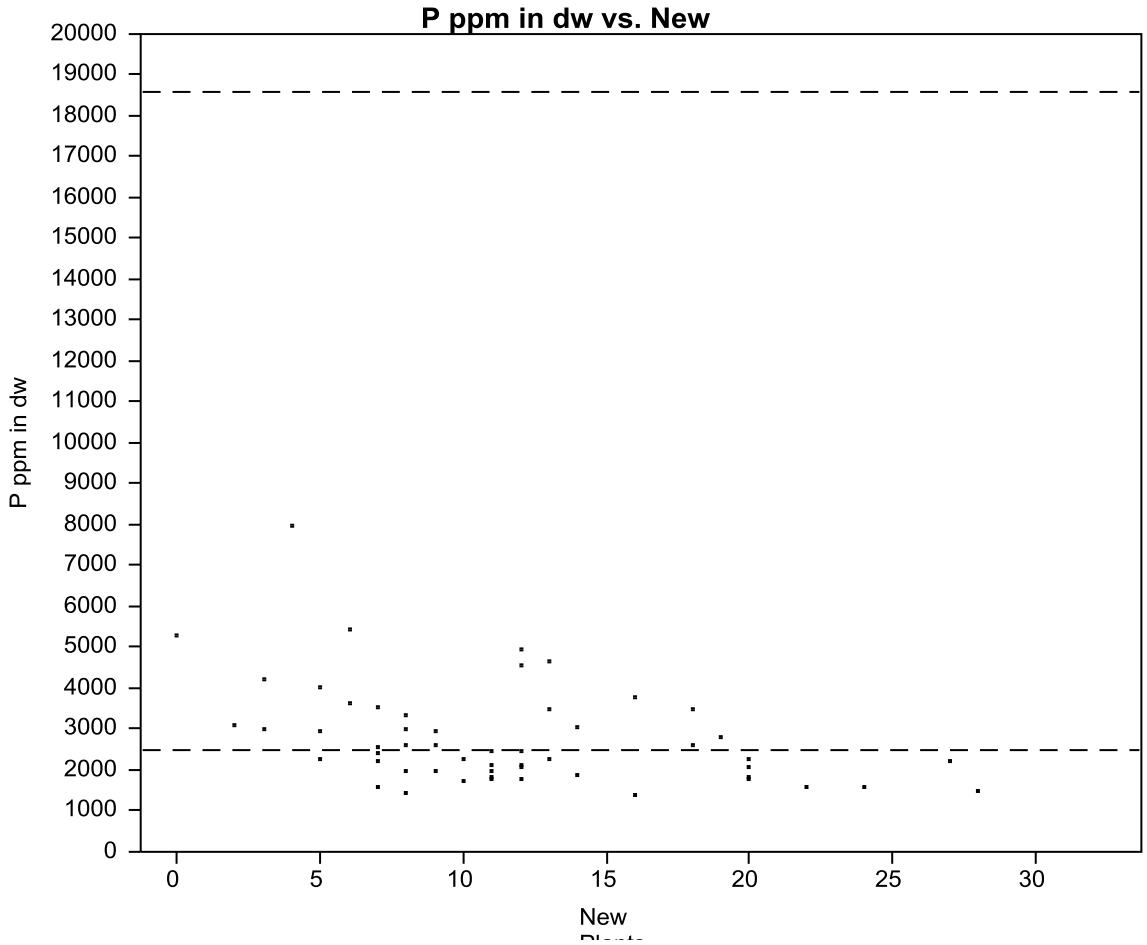

P ppm in dw vs. New
P ppm in dw
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
New

Figure 2.9

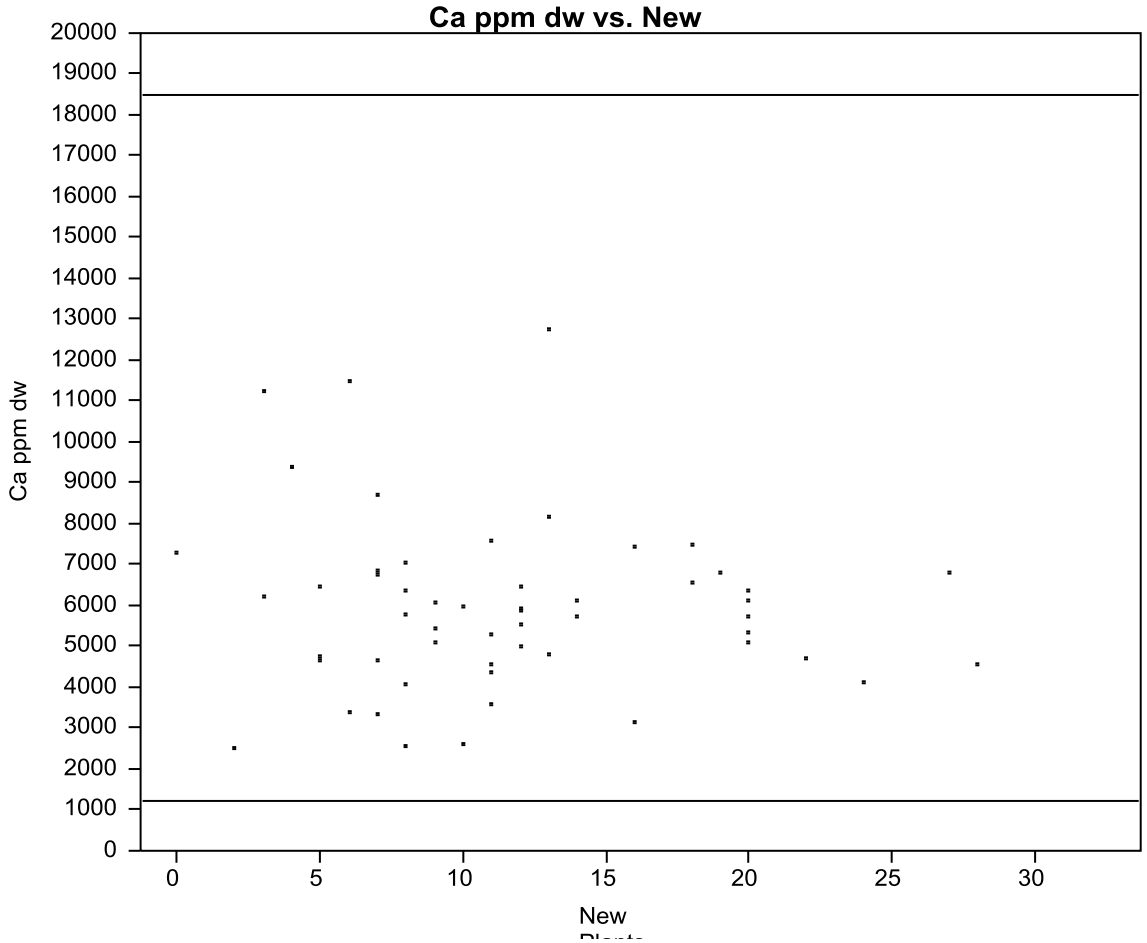

Ca ppm dw vs. New
Ca ppm dw
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
New

Figure 2.10

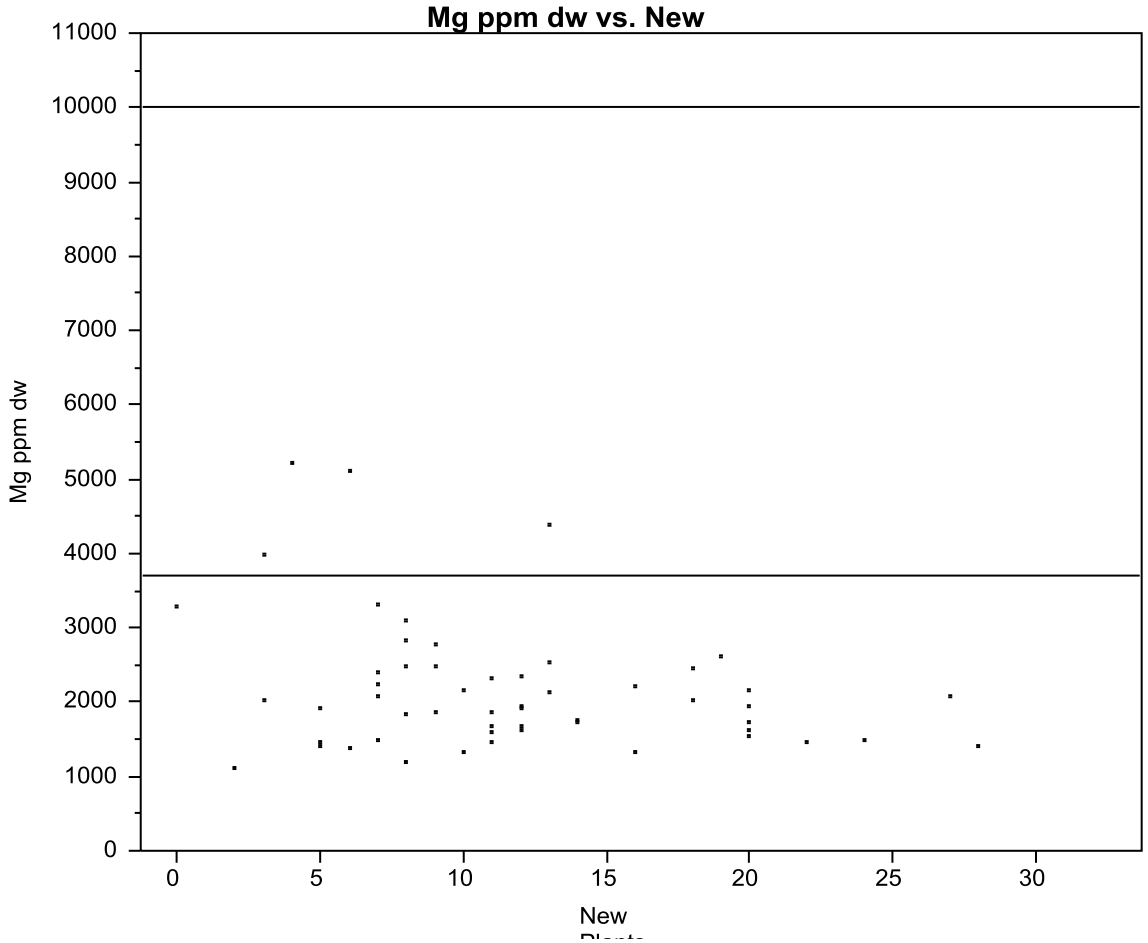
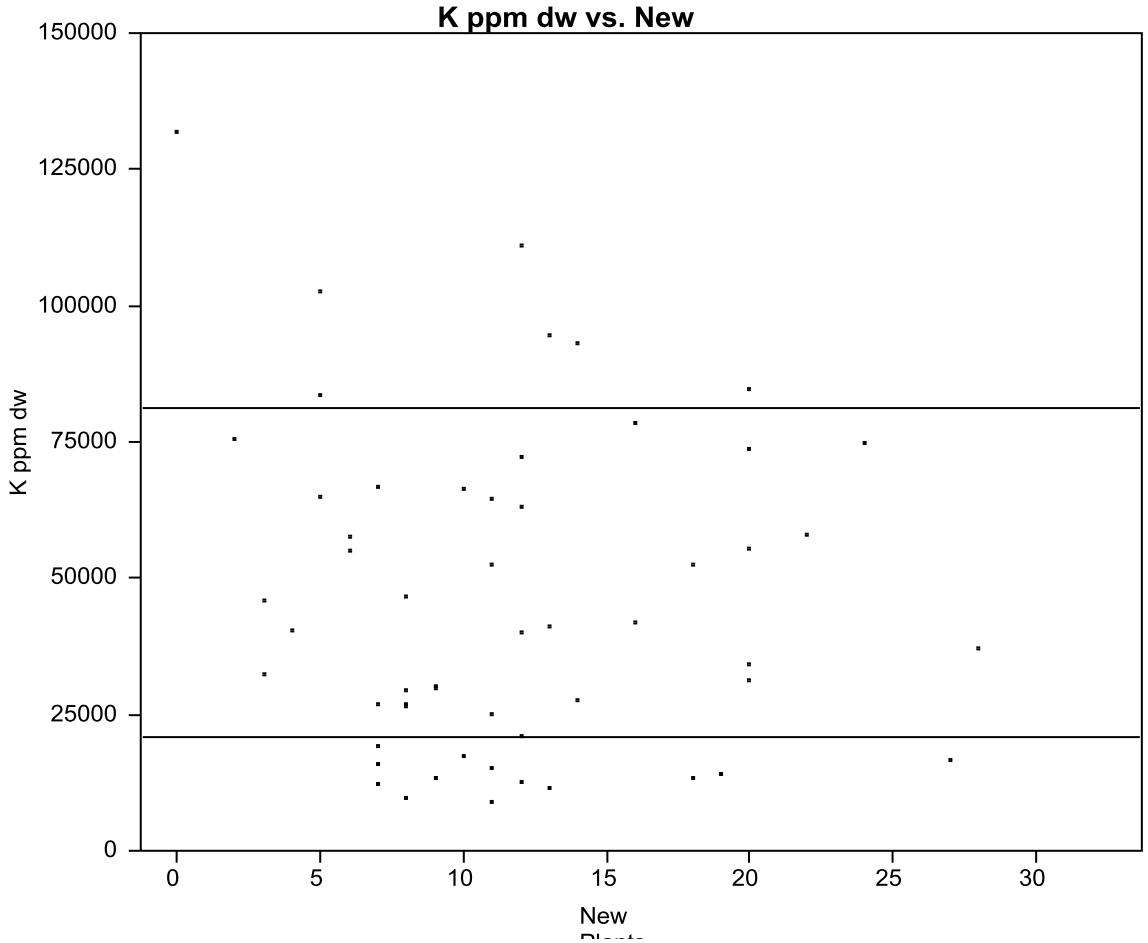

Mg ppm dw vs. New
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Mg ppm dw
0
5
10
15
20
25
30
New

Figure 2.11


K ppm dw vs. New
150000
125000
100000
75000
50000
25000
0
K ppm dw
0
5
10
15
20
25
30
New

APPENDICES

Appendix I

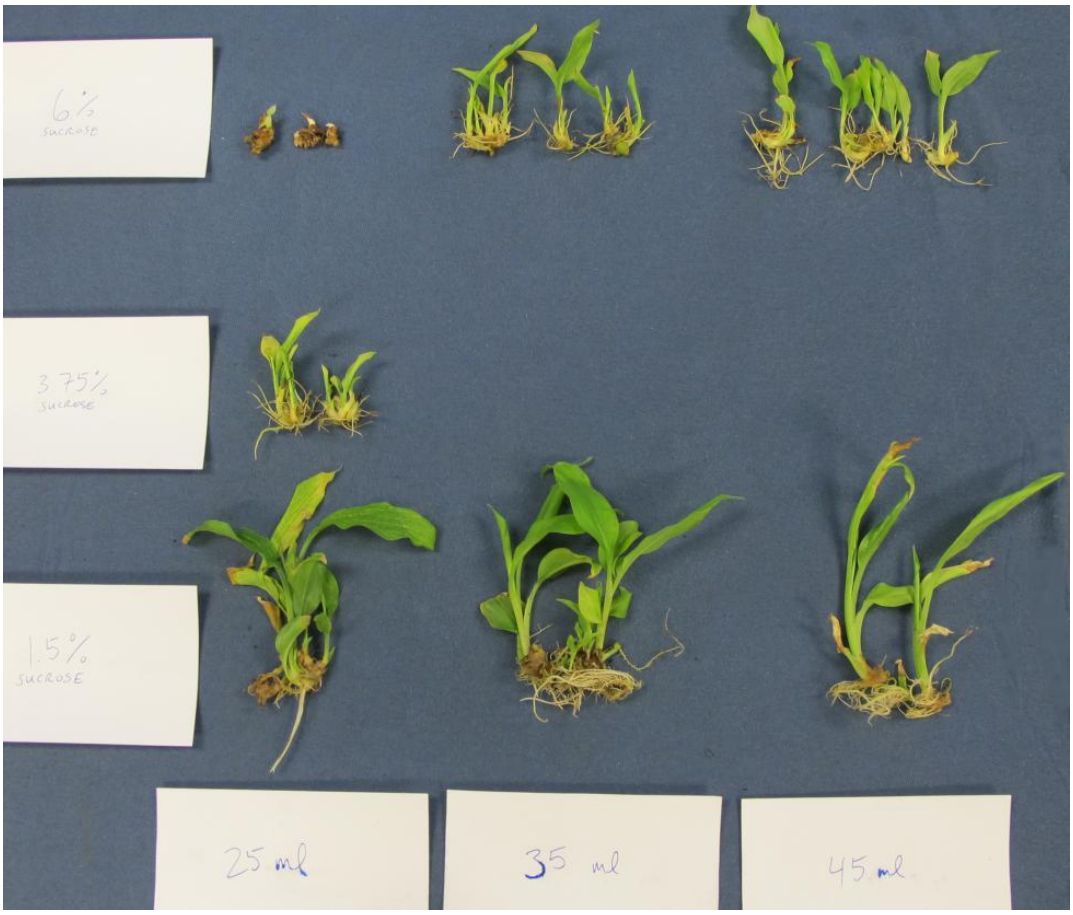
Prediction profiler of Figure 1.2 (B,C) in dynamic format, both .html and .swf file types

Appendix II

Prediction profiler of Figure 1.5 in dynamic format, both .html and .swf file types

Appendix III

Pictoral representation of media volume by sucrose interaction at 3 plants per vessel in experiment 2, to supplement Figure 1.2 (A)



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