The effects of nitrogen and potassium nutrition on the growth of nonembryogenic and embryogenic tissue of sweet orange (Citrus sinensis (L.) Osbeck)

Randall P Niedz* and Terence J Evens

Address: United States Department of Agriculture, Agricultural Research Service, US Horticultural Research Laboratory, Ft. Pierce, FL 34945-3030, USA
Email: Randall P Niedz* - Randall.Niedz@ars.usda.gov; Terence J Evens - Terence.Evens@ars.usda.gov
* Corresponding author

Abstract

Background: Mineral nutrients are one of the most basic components of plant tissue culture media. Nitrogen in the form of NH₄⁺ and NO₃⁻ is the dominant mineral nutrient in most plant tissue culture formulations, with effects dependent on both the proportion and the amount of NH₄⁺ and NO₃⁻. The effects of nitrogen nutrition on the growth of nonembryogenic and embryogenic cell lines of sweet orange (C. sinensis (L.) Osbeck cv. 'Valencia'), tissues routinely used in citrus horticultural and plant improvement research, was explored using an experimental approach free of ion confounding that included a 2-component mixture (NH₄⁺:K⁺) and a quantitative factor [NO₃⁻] crossed by the mixture, thereby providing ion-specific estimates of proportional and amount effects.

Results: First, the linear mixture component, though only a comparison of the design space vertices, was highly significant for both tissue types and showed that NH₄⁺ was required by both tissues. Second, the NH₄⁺:K⁺ mixture term was highly significant for both tissue types, revealing that NH₄⁺ and K⁺ exhibit strong synergistic blending and showed that growth was substantially greater at certain blends of these two ions. Third, though the interaction between the NH₄⁺:K⁺ mixture and NO₃⁻ amount on fresh weight accumulation for both tissue types was significant, it was substantially less than the main effect of the NH₄⁺:K⁺ mixture. Fourth, a region of the design space was identified where fresh weight growth was increased 198% and 67% over the MS medium controls for nonembryogenic and embryogenic tissues.

Conclusion: By designing a mineral nutrient experiment free of ion confounding, a direct estimation of ion-specific proportional and amount effects on plant tissue growth is possible. When the ions themselves are the independent factors and/or mixture components, the resulting design space can be systematically explored to identify regions where the response(s) is substantially improved over current media formulations. In addition, because the response is over a defined experimental region, a specific medium formulation is more accurately interpreted as a coordinate in the specified design geometry.
Background

Mineral nutrients are one of the most basic components of plant tissue culture media. Unlike carbon sources, plant growth regulators, vitamins, amino acids, gelling agents and undefined substances that may or may not be included in any given medium, the mineral nutrients are always present [1]. Thus, a great deal of time and effort has been devoted to identifying the optimal concentrations for each of the currently established 14 essential plant nutrients [2]. Nitrogen in the form of NH4+ and NO3- is the dominant mineral nutrient in most tissue culture formulations including MS [3] the most widely used nutrient formulation in plant tissue culture. Nitrogen effects are highly dependent on both the total amount of nitrogen and on the proportion of NH4+ and NO3- and affect a wide range of in vitro responses including callus growth, shoot and root organogenesis, embryogenesis, and shoot multiplication [1]. We thus chose to determine the effects of nitrogen nutrition on the growth of nonembryogenic and embryogenic cell lines of sweet orange (C. sinensis (L.) Osbeck).

Nonembryogenic tissue has been used for biochemical characterization of pathogenesis-related (PR) proteins [4] and as a source of protoplasts for somatic hybridization [5]. Embryogenic tissue is often used for enzymatic studies [6,7] is the primary source of protoplasts for somatic hybridization [8] and is also used for genetic transformation [9].

A primary consideration in quantifying the effects of specific mineral nutrients is the concept of ion confounding as previously discussed in [10] and [11]. Ion confounding occurs when salts are treated as experimental factors in experimental designs focused on determining the effects of nutrients/ions in solution. To illustrate this concept, consider a simple experiment wherein a single salt such as KNO3 is varied over some concentration range and a particular in vitro response is measured. Any measured change in the response may be due to K+, NO3-, and/or the interaction between K+ and NO3-. When salts are used as factors both ions are simultaneously varied; consequently, their effects are potentially confounded with each other [12,13]. No valid conclusions can be derived regarding the main effects of the two component ions K+ or NO3- or their interaction from such an experiment. The measured effect in a salt-based experiment is actually the mean effect of the two ions, K+ and NO3-, in a 1:1 proportion at varying concentrations. The so-called “co-ion approach” often employed to circumvent this limitation is not valid [11]. In short, ion confounding occurs when the ion(s) of interest are covaried with the complementary ion(s) associated with the salts used; that is, attempting to vary the concentration of a single cation or anion using a salt results in a simultaneous change in the associated co-ion. Such changes also include ions added via pH adjustments but unaccounted for in the experimental design. We report the results from an approach that, to the best of our knowledge, is the first study on the effects of nitrogen nutrition obtained with experimentation free of ion confounding.

Results

Nonembryogenic callus

The percentage increase of the fresh weight of nonembryogenic (NE) sweet orange callus over fourteen days ranged from 2% – 926% (Table 1), indicating that K+, NH4+, and NO3- nutrition are important regulators of NE callus fresh weight growth. For % fresh weight increase the best fitting polynomial was a reduced quadratic mixture × cubic process response surface obtained by backward elimination. A summary of the ANOVA, lack-of-fit test and three R2 statistics for % fresh weight increase and dry weight are presented in Table 2. A single point (Table 1: #14) was identified as suspect by the outlier-t test [14] and was ignored in the fresh and dry weight analyses.

Fresh weight growth (Fig. 1A) required a square root transformation as per a Box-Cox power transform plot analysis. The residual and model diagnostics were within acceptable limits [15]. The lack-of-fit test was not significant (p = 0.2641) indicating that additional variation in the residuals could not be removed with a better model. R2, R2adj and R2pred statistics ranged from 0.95 – 0.98. The overall model was highly significant (p < 0.0001), indicating NH4+, K+, and NO3- significantly affected growth. The ANOVA contained seven significant terms; three of the terms, the linear mixture, NH4+ × K, and NH4+ × K × [NO3]-, had highly significant p-values (i.e. < 0.0001; Table 2).

Dry weight accumulation (Fig. 2A) ranged from 0.04 g – 0.27 g and required a log base 10 transformation as per a Box-Cox power transform plot analysis. Model diagnostics were within acceptable limits and the lack-of-fit test was not significant (p = 0.3634), indicating that additional variation in the residuals could not be removed with a better model. R2, R2adj and R2pred statistics ranged from 0.78 – 0.92, indicating good agreement between these three values. The overall model was highly significant (p < 0.0001) indicating significant factor effects on dry weight by these three ions. The ANOVA revealed three significant terms; two of which, the linear mixture and NH4+ × K terms, had p-values < 0.0001 (Table 2).

Embryogenic callus

The percentage increase of the fresh weight of embryogenic (E) sweet orange callus over fourteen days ranged from 23% – 539% (Table 1), indicating that K+, NH4+, and NO3- nutrition are important regulators of this response. For % fresh weight increase, the best fitting polynomial was a reduced quadratic × cubic (mixture ×
numeric factor) response surface obtained by backward elimination. A summary of the ANOVA, lack-of-fit test and three $R^2$ statistics are presented in Table 3. Fresh weight growth data required a square root transformation as per a Box-Cox power transform plot analysis. Model diagnostics were within acceptable limits and the lack-of-fit test was not significant ($p = 0.3024$) indicating that additional variation in the residuals could not be removed with a better model. The three $R^2$ statistics ranged from 0.8 – 0.98. The overall model was highly significant ($p < 0.0001$), indicating NH$_4^+$, K$^+$, and NO$_3^-$ significantly affected growth. The ANOVA revealed six significant terms; two of which, the linear mixture and NH$_4^+$ * K$^+$ had highly significant p-values (Table 3). Fresh weight growth over the design space is shown in Fig. 1B.

Dry weight accumulation (Fig. 2B) required a log base 10 transformation as per a Box-Cox power transform plot analysis. Model diagnostics were within acceptable limits and the lack-of-fit test was significant ($p = 0.0036$) indicating that 1) additional variation in the residuals might be accounted for with a better model or, 2) an unusually low level of pure error was present. The three $R^2$ statistics ranged from 0.89 – 0.96. The overall model was highly significant ($p < 0.0001$) indicating significant factor effects on dry weight accumulation by these three ions. The ANOVA revealed four significant terms, three of which, the linear mixture, NH$_4^+$ * K$^+$, and NH$_4^+$ * [NO$_3^-$] had p-values < 0.0001 (Table 3).

### Table 1: Mixture-amount treatment points and fresh- and dry-weight data for NE (nonembryogenic callus) and E (embryogenic callus).

| Treatment Design Points | Block | Mixture Components | Factor | Fresh Wgt (Increase %) | Dry Wgt (g) | pH$^+$ |
|-------------------------|-------|--------------------|--------|------------------------|-------------|--------|
|                         |       | NH$_4^+$ | K$^+$ | NO$_3^-$ mM | NE | E | NE | E | NE | E |
| 1                       | 1     | 0.250   | 0.750 | 10.00 | 387 | 288 | 0.141 | 0.183 | 5.7 |
| 2                       | 1     | 0.500   | 0.500 | 50.00 | 329 | 344 | 0.125 | 0.243 | 5.8 |
| 3                       | 1     | 0.500   | 0.500 | 30.00 | 317 | 539 | 0.127 | 0.176 | 5.8 |
| 4                       | 1     | 0.000   | 1.000 | 20.00 | 13  | 33  | 0.066 | 0.074 | 5.7 |
| 5                       | 1     | 0.500   | 0.500 | 10.00 | 408 | 315 | 0.140 | 0.146 | 5.7 |
| 6                       | 1     | 0.250   | 0.750 | 30.00 | 926 | 520 | 0.266 | 0.262 | 5.8 |
| 7                       | 1     | 0.250   | 0.750 | 30.00 | 885 | 490 | 0.246 | 0.256 | 5.8 |
| 8                       | 1     | 0.250   | 0.750 | 50.00 | 425 | 313 | 0.152 | 0.252 | 5.8 |
| 9                       | 1     | 0.500   | 0.500 | 30.00 | 406 | 391 | 0.143 | 0.154 | 5.8 |
| 10                      | 1     | 0.000   | 1.000 | 50.00 | 15  | 33  | 0.059 | 0.062 | 5.8 |
| 11                      | 1     | 0.250   | 0.750 | 10.00 | 360 | 332 | 0.160 | 0.208 | 5.7 |
| 12                      | 1     | 0.500   | 0.500 | 50.00 | 290 | 333 | 0.122 | 0.244 | 5.8 |
| 13                      | 1     | 0.000   | 1.000 | 50.00 | 13  | 33  | 0.066 | 0.061 | 5.8 |
| 14                      | 2     | 0.500   | 0.500 | 10.00 | 406 | 52  | 0.149 | 0.068 | 5.7 |
| 15                      | 2     | 0.000   | 1.000 | 10.00 | 10  | 34  | 0.058 | 0.092 | 5.7 |
| 16                      | 2     | 0.125   | 0.875 | 30.00 | 191 | 295 | 0.106 | 0.245 | 5.8 |
| 17                      | 2     | 0.500   | 0.500 | 40.00 | 99  | 298 | 0.073 | 0.245 | 5.8 |
| 18                      | 2     | 0.250   | 0.750 | 50.00 | 115 | 280 | 0.080 | 0.234 | 5.8 |
| 19                      | 2     | 0.250   | 0.750 | 50.00 | 205 | 282 | 0.110 | 0.242 | 5.8 |
| 20                      | 2     | 0.500   | 0.500 | 40.00 | 123 | 308 | 0.079 | 0.259 | 5.8 |
| 21                      | 2     | 0.500   | 0.500 | 10.00 | 208 | 202 | 0.107 | 0.12  | 5.7 |
| 22                      | 2     | 0.000   | 1.000 | 40.00 | 2   | 23  | 0.044 | 0.071 | 5.8 |
| 23                      | 2     | 0.000   | 1.000 | 10.00 | 12  | 24  | 0.056 | 0.084 | 5.7 |
| 24                      | 2     | 0.000   | 1.000 | 10.00 | 9   | 33  | 0.050 | 0.096 | 5.7 |
| 25                      | 2     | 0.250   | 0.750 | 20.00 | 403 | 493 | 0.156 | 0.241 | 5.8 |
| 26                      | 2     | 0.000   | 1.000 | 40.00 | 15  | 71  | 0.0702| 0.095 | 5.8 |

Experiment is a two-component mixture of NH$_4^+$ and K$^+$ and one quantitative factor NO$_3^-$ amount. The mixture components are listed as proportions with their actual amounts matched to the amount of NO$_3^-$. For example, treatment point #1 included 2.5 mM NH$_4^+$, 7.5 mM K$^+$, and 10 mM NO$_3^-$. The data represent the mean of six duplicate plates per treatment point. Points #17 and #20 are MS medium.

---

**Analysis**

The effects of these three ions on nonembryogenic and embryogenic tissue growth were similar in several ways. First, the linear mixture component was highly significant for both tissue types. The linear mixture component compares the responses at the extreme ends (vertices) of the mixture design space. This means that growth at the points comprising the 0 NH$_4^+$:1 K$^+$ ratio were compared to growth at the points comprising the 0.5 NH$_4^+$:0.5 K$^+$ ratio. Likewise, the regression coefficients for NH$_4^+$ and K$^+$ in

* -- Calculated from the chemical equilibrium modeling software MINEQL+ Ver. 4.5 (27), temperature corrected and assumed open to the atmosphere with a $P_{CO2}$ at sea level of 10$^{-3.50}$ atm.
Tables 2 and 3 are estimates of growth at the vertices only, not estimates of the effects of these components. It is clear when viewing Figure 1 that growth along the 0 NH₄⁺:1 K⁺ y-axis is considerably less than growth along the 0.5 NH₄⁺:0.5 K⁺ y-axis; this is reflected in the larger regression coefficient for NH₄⁺ vs. K⁺ in Tables 2 and 3. Good growth of citrus nonembryogenic and embryogenic tissue requires that [NH₄⁺]>0, which is consistent with many other tissue culture systems [1].

Second, the NH₄⁺ *K⁺ term was highly significant for both tissue types (Tables 2, 3), which reveals that NH₄⁺ and K⁺ exhibit strong synergistic blending. This means that growth was substantially greater at certain blends of these two ions vs. the growth that was observed at the extreme ends or vertices of the mixture. For the two cell lines, ratios from 0.250 NH₄⁺:0.750 K⁺ to 0.375 NH₄⁺:0.625 K⁺ resulted in the greatest increase in fresh weight (Figs. 1, 2). These ranges correspond to a NH₄⁺:NO₃⁻ ratio of 1:3 at 37.5 mM total N at the centerpoints (i.e., the points where the greatest growth was recorded) – note that in standard MS medium this ratio is 1:2 at 60 mM total N (Figs. 1, 2).

It should be noted that the NH₄⁺:NO₃⁻ ratio effect is only correlative and cannot be directly quantified from our experimental design.

Third, the effect of the NH₄⁺:K⁺ mixture and NO₃⁻ amount on dry weight accumulation was comparable to fresh weight accumulation of nonembryogenic callus. Specifically, the NH₄⁺:K⁺ mixture was the primary driver of dry weight accumulation. One difference was that NO₃⁻ amount had less of an effect on dry weight than it did for fresh weight accumulation. This result possibly suggests that the NH₄⁺:K⁺ mixture promotes cell division and NO₃⁻ amount promotes cell expansion. Resolution of this effect cannot be done with the experimental design used in this study. To do this would require that all proportion effects be accounted for in an NH₄⁺:K⁺:NO₃⁻ mixture-amount design, which would capture the two currently “hidden” two- and three-component effects, namely, K⁺:NO₃⁻, NH₄⁺:NO₃⁻, and NH₄⁺:K⁺:NO₃⁻. For embryogenic callus the results were somewhat different; there was a relatively strong NH₄⁺ * NO₃⁻ amount effect on dry weight not observed for fresh weight (Figure 1B vs. 2B).

### Table 2: ANOVA, regression coefficients, and summary statistics for percentage fresh weight increase and dry weights of nonembryogenic tissue.

| Source | Fresh Wgt.a | Dry Wgt.a |
|--------|-------------|-----------|
|        | F Value     | p-values  | Regression coefficients<sup>c</sup> | F Value     | p-values  | Regression coefficients<sup>c</sup> |
| Model  | 87.23       | < 0.0001  |                       | 22.18       | < 0.0001  |                       |
| Linear Mixture | 279.34       | < 0.0001  |                       | 50.77       | < 0.0001  |                       |

| NH₄⁺ | 15.24 |                       |                       |
| K⁺   | 2.63  |                       |                       |

| NH₄⁺ * K⁺ | 290.91 | < 0.0001  | + 70.70  | 64.35 | < 0.0001  | + 1.60 |
| NH₄⁺ * [NO₃⁻] | 7.72    | 0.0148 | - 1.72 | 2.53 | 0.1326 | - 0.049 |
| K⁺ * [NO₃⁻] | 15.50   | 0.0015 | + 8.34 | 1.49 | 0.2409 | - 0.035 |
| NH₄⁺ * K⁺ * [NO₃⁻] | 10.37   | 0.0062 | + 9.99 | 0.54 | 0.4754 | + 0.11 |
| NH₄⁺ * [NO₃⁻]² | 0.16 | 0.6979 | + 0.41 | 0.20 | 0.6593 | + 0.023 |
| K⁺ * [NO₃⁻]² | 0.53 | 0.4771 | + 0.93 | 0.29 | 0.5950 | - 0.034 |
| NH₄⁺ * K⁺ * [NO₃⁻]² | 70.53   | < 0.0001  | - 44.15 | 12.10 | 0.0034 | - 0.89 |
| K⁺ * [NO₃⁻]³ | 24.16 | 0.0002 | - 11.45 | - | - |

| Lack of Fit | p = 0.2641 | p = 0.3634 | |
| R² | 0.98 | 0.92 | |
| R² adj | 0.97 | 0.88 | |
| R² pred | 0.95 | 0.78 | |
| Std. Dev. | 1.28 | 1.28 | |
| Mean | 13.32 | 13.32 | |
| C.V. % | 9.63 | 6.33 | |
| Model type | reduced quadratic × cubic<sup>b</sup> | quadratic × quadratic | |

<sup>a</sup> Data transformation was determined by a Box Cox plot analysis – fresh weight data were transformed by square root and NE dry weight by log base 10.

<sup>b</sup> Model reduction by backward elimination.

<sup>c</sup> Presented in coded form. Coding normalizes the factor ranges by placing their low and high range value between -1 and +1 and can thus, be directly compared.
Figure 1
Fresh and dry weight response contour plots for nonembryogenic tissue. A) % increase in fresh weight growth; B) dry weight. Pictures of the difference in biomass between standard MS and the center point of the experimental design space are pictured to the right of each plot.
Fresh and dry weight response contour plots for embryogenic tissue. A) % increase in fresh weight growth; B) dry weight. The standard MS point and the point of greatest growth are indicated on each plot.
Interestingly, the interaction between the NH$_4$+K+ mixture and NO$_3$- amount for fresh weight growth as revealed in the NH$_4$+ K+ [NO$_3$] and NH$_4$+ K+ [NO$_3$]$_2$ terms was significant for the nonembryogenic tissue, but not significant for the embryogenic callus. This probably reflects the greater effect of these factors on nonembryogenic tissue where the point of greatest growth was a 198% increase in fresh weight vs. a 67% increase for embryogenic tissue. It should be pointed out that the magnitude of the effects of the interaction between NH$_4$+K+ mixture and NO$_3$- amount on fresh weight accumulation for both tissue types was substantially less than the main effects of the NH$_4$+K+ mixture – evident when the p-values are compared.

### Discussion

The effects of NH$_4$+, K+, and NO$_3$- on the growth of non-embryogenic and embryogenic citrus callus were determined using an approach that removed ion confounding from the experimental design. The basic approach was to 1) design an experiment where the ions NH$_4$+, K+, and NO$_3$- as opposed to their salts [16], were the factors to be varied [11]; 2) fix all other inorganic ions at their MS levels; 3) calculate the salt/acid/base formulations required to achieve the ion levels specified for each treatment combination using the ion/salt linear programming algorithm and the software ARS-Media [10] to remove ion confounding. Because mineral nutrients are known to include both proportional and amount effects, proportionality and amount were incorporated into the design of the experiment. Because the three ions are all monovalent, treating NH$_4$+ and K+ as a 2-component mixture matched to the amount of NO$_3$ resulted in a design space of near uniform pH. The effects of total nitrogen and the ratio of NH$_4$+ to NO$_3$ were indirectly captured in the selected design. It is important to point out that this approach did not directly control pH, i.e. treat pH as an independent variable. All the bulk solution properties of the initial media solutions such as pH and ion speciation were inherent to the selected design space. Because the bulk solution properties were treated as dependent variables, the experiment was free of ion confounding and allowed estimation of ion-specific effects on tissue growth. However, it is important to note that by 'constraining' pH in

---

**Table 3: ANOVA, Regression coefficients, and summary statistics for percentage fresh weight increase and dry weights of embryogenic tissue.**

| Source | Embryogenic tissue | Fresh Wgt. | Dry Wgt. |
|--------|-------------------|------------|----------|
|        | F Value | p-values | Regression coefficients | F Value | p-values | Regression coefficients |
| Model  | 59.82   | < 0.0001 | 49.07 | < 0.0001 |
| Linear Mixture | 350.72 | < 0.0001 | 153.04 | < 0.0001 |
| NH$_4$+ | + 21.15 | + 0.19 |
| K+     | + 6.01  | + 0.085 |
| NH$_4$+ K+ | 73.69 | < 0.0001 | 86.33 | < 0.0001 |
| [NO$_3$] | + 36.18 | + 0.54 |
| K+ [NO$_3$] | 6.86 | 0.0390 | 3.20 | 0.0001 |
| NH$_4$+ K+ [NO$_3$] | 0.42 | 0.1471 | 0.65 | 0.4339 |
| NH$_4$+ [NO$_3$]$_2$ | 15.18 | 0.0018 | 0.24 | 0.6279 |
| K+ [NO$_3$]$_2$ | 0.11 | 0.7409 | 0.27 | 0.6102 |
| NH$_4$+ K+ [NO$_3$]$_2$ | 5.76 | 0.0321 | 0.92 | 0.0188 |
| NH$_4$+ [NO$_3$]$_3$ | 6.96 | 0.0205 | + 7.95 | - |
| K+ [NO$_3$]$_3$ | 2.23 | 0.1596 | - 3.35 | - |

Lack of Fit | p = 0.3024 | p = 0.0036 |
| R$^2$ | 0.98 | 0.96 |
| R$^2$ adj | 0.96 | 0.94 |
| R$^2$ pred | 0.80 | 0.89 |
| Std. Dev. | 1.24 | 0.018 |
| Mean | 14.53 | 0.17 |
| C.V. % | 8.52 | 10.59 |

Model type | reduced quadratic × cubic$^b$ | quadratic × quadratic |

---

$^a$ Data transformation was determined by a Box Cox plot analysis – fresh weight data were transformed by square root and NE dry weight by log base 10.

$^b$ Model reduction by backward elimination.

$^c$ Presented in coded form. Coding normalizes the factor ranges by placing their low and high range value between -1 and +1 and can thus be directly compared.
this manner we were unable to directly quantify the effects of the NH$_4^+$:NO$_3^-$ ratios, i.e. we can only perform correlational analyses to explore these effects.

We assumed when designing the experiment for this study that most of the K$^+$ ions would primarily affect bulk solution properties (as opposed to the μmolar amounts required to meet tissue nutritional requirements) such as the electrical charge of the system. The experimental design space explored in this study is actually a subset of points that describe a plane through a less-constrained 3-dimensional design space defined by the axes NH$_4^+$:K$^+$, NH$_4^+$:K$^+$ amount, and NO$_3^-$ amount (Fig. 3). All of the points falling on this plane have a pH near 5.8 – pH increases above the plane and decreases below it. This raises the question of the importance of the starting pH of the culture medium. Given that tissue growth was not uniform across the experimental design space, tissue growth did not correlate well with initial solution pH. The nonuniform growth across a common pH plane is analogous to the surprisingly low correlation between solution pH and protein precipitation observed in experiments free of ion confounding designed to detect ion-specific effects [11]. For the responses measured in this study, the primary drivers are the ions under independent control (i.e. NH$_4^+$, K$^+$ and NO$_3^-$) and is an empirical demonstration that pH is a dependent variable and must be treated as such experimentally. The pH, or 'relative proton activity', of a given solution is primarily determined by the type and concentration of the ions in solution. Thus, pH can only be examined in a correlational relationship and cannot be established as a causal factor. One implication of these results is that an experimental design to grow plant tissues on mineral nutrient combinations free of ion confounding greatly expands the experimental design space by removing the "pH bias" (Fig. 3). Are there regions in the experimental design space described in Figure 3 where citrus tissue would grow as well or better than on the plane sampled in these experiments? Given that each of the responses displayed a 'hot-spot' near the center of the experimental design space, there is no reason to assume that there may not be even better regions for growth that lie above or below this plane. Certainly, by sampling the full cubic design space depicted in Figure 3, regions outside of pH 5.8 where citrus tissue grows well might be identified. Thus, the selection of the "pH 5.8" plane was, to some extent arbitrary, and was chosen because it was the pH value used by Murashige and Skoog [3]. The value of the experiments conducted by Murashige and Skoog was that most of the components required for culturing a wide range of plant species in vitro were identified, and a formulation was developed that works in some fashion for a large variety of plant species. MS has been very useful as a starting point for species-specific media formulation optimization studies. The fact that the best media formulation for citrus tissue growth differs from MS is not surprising given that MS medium was developed for tobacco pith callus as opposed to citrus callus. However, because MS was developed using salt-based, pH-adjusted, one-factor-at-a-time (OFAT) experiments, we really have no reason to assume that MS is optimal even for tobacco pith callus. The research presented here is a logical extension of Murashige and Skoog's seminal work, and represents the next step in the evolution of plant tissue culture media development.

**Conclusion**

A substantial increase in tissue growth was observed in sweet orange nonembryogenic and embryogenic tissue in certain regions of a 2-dimensional design space defined by 2-component NH$_4^+$:K$^+$ mixture and NO$_3^-$ amount axes. Such an approach removes ion confounding, treats all initial bulk solution properties as dependent variables, and separates proportional and amount effects. The result is an experimental design space defined by ion factors/components suited for systematic exploration. Some of the implications of this approach include 1) the practical aspects of developing improved media formulations; 2) the more basic aspects of quantifying ion-specific responses in an experimentally rigorous manner and
relating these responses changes to gene/protein/metabolite profiles and phenotype and; 3) the concept that specific media formulations are more properly viewed as "ion-coordinates" in a hyperdimensional geometry defined by all of the components that constitute a medium rather than as the salt recipe that is used to create that medium.

Methods

**Plant Material and Tissue**

**Nonembryogenic cell line**

A five year old nonembryogenic cell line was developed from epicotyl explants of vitro grown seedlings of *Citrus sinensis* (L.) Osbeck cv. 'Valencia.' Seed were germinated in MS basal medium without plant growth regulators and supplemented with 3% (w/v) sucrose. One cm epicotyl explants were excised from 15–21 d old seedlings and placed onto MT medium [17] supplemented with 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1 μM 6-benzylaminopurine (BA) and 100 mg L⁻¹ casein hydrolysate. The cultures were grown in a temperature-controlled growth cabinet at 27°C on a 4-h photoperiod under low light (15–20 μmol photons m⁻² s⁻¹) that was provided by cool-white fluorescent lamps. After 6 months of selection, rapidly growing tissue was obtained. For maintenance, the 2,4-D concentration was reduced to 1 μM.

**Figure 4**

Experimental design space with treatment points. NH₄⁺:K⁺ mixture- NO₃⁻ amount design space with contours of the standard error of prediction. The standard error of prediction showed is < 1 across the design space.
Table 4: Ion values (mM) for four treatments, including MS medium, used to solve the linear programming algorithm utilized by ARS-Media.

| Ion            | MS          | 1       | 2       | 3       |
|----------------|-------------|---------|---------|---------|
| B(OH)3         | 0.100259    | 0.100259| 0.100259| 0.100259|
| Ca(2+)         | 2.992884    | 2.992884| 2.992884| 2.992884|
| Cl(-)          | 5.985982    | 5.985982| 5.985982| 5.985982|
| Co(2+)         | 0.000105    | 0.000105| 0.000105| 0.000105|
| Cu(2+)         | 0.0001      |         | 0.0001  |         |
| EDTA           | 0.100027    | 0.100027| 0.100027| 0.100027|
| Fe(2+)         | 0.099997    | 0.099997| 0.099997| 0.099997|
| I(-)           | 0.004999    | 0.004999| 0.004999| 0.004999|

The results are the salt/acid/base formulations required to make each treatment solution. All ions other than the three being varied (italicized and bold) are fixed and unvaried, which illustrates an experimental design free of ion confounding.

Embryogenic cell line

A three year old embryogenic callus line derived from C. sinensis cv. 'Valencia' was initiated as described by [18]. The line was maintained on Murashige and Tucker's (MT) basal medium [17] at 27°C, in the dark, and on a 28-d subculture cycle.

To acclimate the tissue to each test formulation and minimize possible carry-over effects, experiments were initiated by first culturing approximately 1 g of callus onto each treatment formulation (or "design point"), using 100 × 15 mm polystyrene culture dishes, followed by two additional transfers. The result was that prior to experimentation the tissue used was acclimated to each treatment formulation for three fourteen day growth cycles. Following the acclimation cycles, approximately 1 g from the acclimated cultures was subcultured again onto each treatment formulation and allowed to grow for 14 days before the biomass was harvested. Fresh and dry weights were quantified by taking the average of six pseudoreplicates for each treatment point. Percent increase in fresh weight was calculated using the initial subcultured weight of the callus.

Experimental Approach, Design, and Analysis

The experiment was designed as a mixture-amount [19,20] and included two mixture components, K+ and NH4+, and one numeric factor, NO3- concentration. Because K+ and NH4+ were treated as components of a mixture, the range for each component is expressed as a proportion; all component proportions in each mixture sum to one. NO3- concentration ranged from 10 to 50 mM, K+ proportion ranged from 0.5 to 1.0 and NH4+ proportion ranged from 0.0 to 0.5. The concentration of K+ plus NH4+ was matched to the NO3- concentration to maintain charge neutrality. No pH adjustments were required since pH was uniform across formulations. Design points were selected using D-optimal criteria to satisfy a quadratic polynomial for the mixture (NH4+:K+) and the numeric factor, [NO3-] crossed by the mixture; the resulting design space is depicted in Figure 4. The experiment included 8 model points, 5 lack-of-fit points, 13 points to estimate pure error, and a point for MS basal medium. The experiment included two blocks to account for the number of treatments that could be managed at one time; several treatments were repeated across the two blocks to provide estimates of block effects.

All solution recipes were derived using the linear programming approach described by [10]. The salts/acid/bases required to make each point in the design space was calculated using ARS-Media (Ver. 1.0) ion solution calculation software, which is available as a free download via http://www.ars.usda.gov/services/software/download.htm?softwareid=148, a software application specifically designed for these types of calculations [10]. For each treatment, all ions present and their amounts were entered into ARS-Media. Ions other than those being varied were fixed at their MS levels. Table 4 illustrates four examples, including MS medium, of the ion types and concentrations that were entered into ARS-Media. Preliminary tests showed that once all the organics and growth regulators were added, 3 mM Na+ was required to bring the pH of the medium to 5.8. Therefore, we added 3 mM Na+ to the 0.202 mM Na+ already present in MS for a total of 3.202 mM. Thus, the resulting formulations calculated by ARS-Media did not require any pH adjustment as the correct amount of Na+ was already incorporated into each formulation.

The software application Design-Expert* 7 (Stat-Ease, Inc, Minneapolis, MN) was used for experimental design construction, model evaluation, and all analyses. Detailed descriptions of the statistical methods used to analyze the data can be found in Niedz and Evens [16] and Evens et al. [21]. Briefly, all possible models from the mean to cubic polynomial were calculated with Design Expert*. Initial model selection was based on a battery of adequacy tests [15]. Normality and constant variance were determined graphically; a Box-Cox plot was used to choose the correct transformations [22]. Overly influential data points were identified with DFITS and DFBETAS plots [23]. Adequate precision of the model was determined by comparing the range of the predicted values at the design
points (y) to the average variance (V-bar) of the prediction [15]. Potential outlier points were checked with externally studentized "outlier-t" [14,24] and Cook’s Distance [25] graphical plots. R², adjusted-R² (R²adj), and predicted-R² (R²pred) were estimated for each selected model [26]. ANOVA calculations were conducted for fresh and dry weight responses of both tissue types. The chemical equilibrium modeling software MINELQ+Ver. 4.5 [27] was used to verify the pH of the treatment solutions. All calculations were temperature corrected and assumed open to the atmosphere with a P CO₂ at sea level of 10⁻³.50 atm. The software application Euler 3D ver. 3.1 [28] was used to construct Figure 3.

Authors’ contributions

RPN conceived and coordinated the study. RPN and TJE jointly developed the experimental design, analyzed and interpreted the data. RPN drafted the manuscript. RPN and TJE edited and approved the final manuscript.

Acknowledgements

We thank Mr. Eldridge Wynn for his careful preparation of the media formulations, growth of the tissue lines, and setup and collection of the data for this study.

References

1. George EF, de Klerk G-J: The components of plant tissue culture media I: macro- and micro-nutrients. In Plant propagation by tissue culture 3rd edition. Edited by: George EF, Hall MA, de Klerk G-J. Dordrecht, The Netherlands: Springer; 2008:65-113.
2. Marschner H: Mineral nutrition of higher plants London: Academic Press; 2003.
3. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962, 15:473-497.
4. Osswald WF, Shapiro JP, Doostdar H, McDonald RE, Niedz RP, Nairn Cj, Hearn Cj, Mayer RT: Identification and characterization of acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissue. Plant Cell Physiol 1994, 35:811-820.
5. Grosser JW, Gmitter FG Jr, Chandler JL: Intergeneric somatic hybrid plants from sexually incompatible woody: Citrus sinensis and Severinia disticha. Theor Appl Genet 1988, 75:397-401.
6. Mayer RT, McColllum GT, Niedz RP, Hearn Cj, McDonald RT, Berdis E, Doostdar H: Purification and characterization of seven basic endochitinases isolated from cell cultures of Citrus sinensis (L.). Planta 1996, 200(3):289-295.
7. Vu JC, Niedz RP, Yelenosky G: Activities of sucrose-metabolizing enzymes in glycerol-grown suspension cultures of 'Hamlin' orange (Citrus sinensis L. Osbeck). Environmental and Experimental Botany 1995, 35:455-463.
8. Guo WW, Wu RC, Cheng Yj, Deng XX: Regeneration and molecular characterisation of two interspecific somatic hybrids of Citrus for potential rootstock improvement. J Hort Sci Biotech 2008, 83:407-410.
9. Niedz RP, Mckendree WL, Shatters RG Jr: Electroporation of embryogenic protoplasts of sweet orange (Citrus sinensis (L.) Osbeck) and regeneration of transformed plants. In Vitro Cell Dev Biol Plant 2003, 39:586-594.
10. Niedz RP, Evens TJ: A solution to the problem of ion confounding in experimental biology. Nature Methods 2006, 3:417.
11. Evens TJ, Niedz RP: Are Hofmeister Series Relevant to Modern Ion-Specific Effects Research? Scholarly Research Exchange 2008.
12. Fisher RA: The factorial design in experimentation. In The Design of Experiments Edited by: Bennett JH. Oxford: Oxford University Press; 1935:93-94.
13. Fisher RA: Confounding. In The Design of Experiments Edited by: Bennett JH. Oxford: Oxford University Press; 1935.
14. Weisberg S: Applied Linear Regression 2nd edition. Hoboken: John Wiley & Sons, Inc.; 1986.
15. Anderson MJ, Whitchom PJ: RSM simplified: optimizing processes using response surface methods for design of experiments New York, NY: Productivity Press; 2005.
16. Niedz RP, Evens TJ: Regulating plant tissue growth by mineral nutrition. Vitro Cell Dev Biol Plant 2007, 43:370-381.
17. Murashige T, Tucker DPh: Growth factor requirements of citrus tissue culture. Proc 1st Int Citrus Symp 1962, 3:1155-1161.
18. Kobayashi S, Ikeda I, Uchiyama H: Conditions for high frequency embryogenesis from orange (Citrus sinensis Osb.) protoplasts. Plant Cell Tiss Org Cult 1985, 4:249-259.
19. Cornell JA: Experiments with Mixtures: Designs, Models and the Analysis of Mixture Data 3rd edition. New York: Wiley & Sons; 2002.
20. Smith WF: Experimental design for formulation Alexandria, VA: ASA-SIAM; 2005.
21. Evens TJ, Niedz RP, Kirkpatrick GJ: Temperature and irradiance impacts on the growth, pigmentation and photosystem II quantum yields of Haematococcus pluvialis (Chlorophyceae). J Appl Phycol 2008, 20:411-422.
22. Box GEP, Cox DR: An analysis of transformations (with discussion). J Royal Statistical Soc Ser B 1964, 26:211-246.
23. Belsley DA, Kuh E, Welsch RE: Regression Diagnostics: Identifying Influential Data and Sources of Collinearity New York: Wiley & Sons, Inc.; 1980.
24. Myers RH: Classical and Modern Regression with Applications 2nd edition. Boston: PWS-KENT Publishing Co.; 1990.
25. Cook RD, Weisberg S: Residuals and Influence in Regression New York: Chapman and Hall; 1982.
26. Myers RH, Montgomery DC: Response surface methodology: process and product optimization using designed experiments 2nd edition. New York, NY:John Wiley & Sons; 2002.
27. Schecher WD, McAvoy DC: MINELQ+ A Chemical Equilibrium Modeling Software: Version 4.0 for Windows User’s Manual Hallowell, Maine, Environmental Research Software; 1998.
28. Euler 3D [http://www.euler3d.hu/index.php?lang=EN]