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THE EFFECTS OF BENZYLADENINE AND META-TOPOLIN ON IN VITRO SHOOT REGENERATION OF SWEET ORANGE

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ABSTRACT

The effects of 6-benzyladeninepurine (BA) and meta-topolin (*m*T) on shoot quality, numbers of explants that produce buds and/or shoots, and the number of shoots greater than 2 mm from 'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) epicotyl explants were determined. The experiment was designed as a mixture-amount. BA and *m*T were varied proportionally from 0 BA: 1 *m*T to 1 BA: 0 *m*T and the total concentration of cytokinins varied from 1 to 50 µM. The polynomial response models developed for each of the three measured responses were highly significant ($p < 0.0001$) and allowed the accurate determination of the proportional and amount effects of these two cytokinins Proportional effects were either not detected (number of explants w/ shoots/buds and number shoots > 2 mm) or were statistically significant but had minimal biological effect (overall quality). Total concentration of cytokinin (BA or *m*T) in the medium was the primary determinant for all three responses.

Keywords: adventitious shoots, sweet orange, benzyladenine, meta-topolin, cytokinins, plant growth regulators.

INTRODUCTION

Adventitious shoot regeneration from plant tissues and organs is pertinent to many important applications such as induction of somaclonal variation and genetic transformation. Initiation of adventitious meristems and subsequent shoot growth from differentiated tissues requires culturing an explant under defined conditions on an artificial, gellified medium containing the appropriate types and concentrations of mineral nutrients, carbon sources, organic constituents, and plant growth regulators. For shoot regeneration, a high cytokinin to auxin ratio is typically required (Murashige and Skoog, 1962) with the operative types and concentrations of cytokinins and auxins varying between species.

In a wide range of citrus types, 6-benzylaminopurine (BA) is the cytokinin used to initiate shoot organogenesis. It is commonly used either singularly or in combination with NAA (Burger and Hackett, 1986; Costa *et al.*, 2004; Duran-vila *et al.*, 1989; Grinblat, 1972; Kobayashi *et al.*, 2003; Paudyal and Haq, 2000; Perez-Molphi-Balch and Ochoa-Alejo, 1997; Zou *et al.*, 2008). Because small differences in molecular structure can have large effects on plant responses, we compared the activities of BAP with the aromatic cytokinin meta-topolin (*m*T), originally isolated from poplar (Strnad *et al.*, 1997) and closely related to BAP, for inducing adventitious shoots from citrus tissue cultures of the 'Hamlin' sweet orange. 'Hamlin' is the most widely grown sweet orange in Florida, accounting for ca. 44% of the 3.3+ million sweet orange nursery propagations in 2008-2009 (Florida Department of Agriculture and Consumer Services, 2009). When grown in a high humidity and temperature environment 'Hamlin' is a highly productive early season variety that produces commercially valuable fruit for juice.

MATERIALS AND METHODS

Plant material, explant source, and culture conditions

Seeds of 'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) were surface disinfested as follows: after removal of the seed coat the seed were soaked for 30 minutes in a 30% bleach (5.25% w/v sodium hypochlorite) solution with a few drops of Tween 20. Seeds were then rinsed three times with sterile water, allowed to soak for 18 hours in water, "sown" onto the surface of MT medium solidified with 8% (w/v) agar in Magenta GA-7-3 vessels (Magenta Corporation, Chicago, IL), and then incubated in the dark at 27°C for three to four weeks. 1 cm explants were excised from the epicotyl of etiolated seedlings. Shoot regeneration experiments were conducted in growth cabinets equipped with cool-white fluorescent lamps (30-55 µmol photons m^{-2} s^{-1}), at 27°C, with a 16-h photoperiod.

BA and mT experiment

The experiment was designed as a mixture-amount (Cornell, 2002; Smith, 2005) and included two mixture components, BA and *m*T, and one numeric factor, total cytokinin concentration (i.e., [BA + *m*T]). Treatment design points are listed in Table-1. Three responses were measured. i) overall quality (on a scale of 1 to 3), ii) number of explants with buds/shoots, and iii) the number of shoot > 2 mm. Because BA and *m*T were treated as components of a mixture, the range for each component is expressed as a proportion; all component proportions in each mixture formulation sum to one. Both BA and *m*T proportions ranged from 0 to 1 and the total cytokinin concentration ranged from 1 to 50 µM. Design points were selected using modified D-optimal criteria to quantify the coefficients of a quadratic polynomial for the 2-component mixture (BA: *m*T) crossed with the numeric factor, 'cytokinin concentration'. The experiment included 9 model points, 6 lack-of-fit points, 5 points to estimate pure

error, and 2 center points for a total of 22 treatment design points. Responses at each treatment design point were estimated from six 100 x 115 mm culture dishes each containing five epicotyl explants derived from a single seedling. Thus, the experiment utilized 132 culture dishes.

Data analysis

The software application Design-Expert® 7 (Stat-Ease, Inc, Minneapolis, MN) was used for experimental design construction, model evaluation, and analyses. Detailed descriptions of the statistical methods used to analyze the data can be found in (Niedz and Evens, 2008; Evens and Niedz, 2008). Briefly, all possible models from the mean to cubic polynomial were calculated. Initial model selection was based on a battery of model adequacy tests (Anderson and Whitcomb, 2005). Normality and constant variance were determined graphically via normal probability plots of residuals; Box-Cox plots were used to identify, if required, the necessity and type of data transformation (Box and Cox, 1964). Overly influential data points were identified with DFFITS and DFBETAS plots (Belsley *et al*., 1980). Adequate precision of the models were determined by comparing the range of the predicted values at the design points ($\hat{y}$) to the average variance (V-bar) of the prediction (Anderson and Whitcomb, 2005). Potential outlier points were checked with externally studentized "outlier-t" (Weisberg, 1986; Myers, 1990) and Cook's Distance (Cook and Weisberg, 1982) graphical plots. R^2, adjusted-R^2 (R^2_{adj}), and predicted-R^2 (R^2_{pred}), were estimated for each selected model (Myers and Montgomery, 2002).

RESULTS

Three responses were measured - overall quality, number of explants with buds/shoots, and the number of shoot > 2 mm. Overall quality was assessed by a gestalt rating for quality (Niedz *et al*., 2007) and ranged from 1 to 3 (Table-1), indicating that BA and/or *m*T affected overall quality. A summary of the ANOVA, lack-of-fit test, three R^2 statistics, and the polynomial model for overall quality are presented in Table-2 and a contour plot of overall quality across the design space in Figure-3. A reduced linear BA: *m*T mixture x quadratic growth regulator concentration polynomial model was selected as the "best" representation of overall quality. The data were transformed as per a Box Cox plot analysis using a power transformation ($gestalt^{-2.11}$) to stabilize the variance. The resulting model was highly significant ($p < 0.0001$). Residual analyses and diagnostic plots were within acceptable limits, indicating that the assumptions required for a valid ANOVA were not violated and that the resulting model was adequate. The lack-of-fit test was not significant ($p = 0.2878$), which indicates that additional variation in residuals could not be removed with a better model. R^2, R^2_{adj} and R^2_{pred} statistics were 0.95, 0.94, and 0.90, respectively. The ANOVA contained five significant terms - linear mixture, BA*mT, BA*concentration, mT*concentration ^{2}, and BA*concentration ^{2}. A significant linear mixture means that the responses at the two extreme ends (vertices) of the mixture design space are significantly different; it is not a measure of the blending effects of the components BA and *m*T (i.e., their effects are determined by a gradient in a specified direction (Smith, 2005)). In this experiment, the linear mixture term is a comparison of all the treatments where the mixture component ratio is 0 BA: 1 *m*T to those that are 1 BA: 0 *m*T. Though the BA*mT term was significant ($p = 0.001$) and revealed BA:*m*T proportional effects, the BA*concentration term had the greatest effect ($p < 0.0001$) on overall quality. The region of the design space that resulted in the highest overall quality was a band across the design space from approximately 1 µM BA to 25 µM *m*T.

The number of explants with buds/shoots ranged from 4 to 20 (Table-1). A summary of the ANOVA, lack-of-fit test, three R^2 statistics, and the polynomial model for the number of explants with buds/shoots are presented in Table-3 and a contour plot of the relationship of BA and *m*T to the number of explants with buds/shoots produced over the design space in Figure-5. A linear BA:*m*T mixture x linear growth regulator amount polynomial model was selected as the "best" representation of the number of buds/shoots across the design space. The resulting model was highly significant ($p < 0.0001$) and no data transformation was required. Residual analyses and diagnostic plots were within acceptable limits. The lack-of-fit test was not significant ($p = 0.824$) and indicated that additional variation in the residuals could not be removed with a better model. The R^2, R^2_{adj} and R^2_{pred} statistics were 0.81, 0.78, and 0.69, respectively. The ANOVA contained two significant terms - BA*concentration and *m*T*concentration. A greater proportion of the variation was in the BA*concentration term (Table-3) reflecting the greater range in the number of explants with buds/shoots at low vs. high concentrations of BA compared to the response range for concentration of *m*T. No BA:*m*T proportional effects were detected. The region of the design space that resulted in the greatest number of buds/shoots was at low levels (< 13.35 µM) of BA.

The number of shoots > 2 mm ranged from 0 to 35 (Table-1). A summary of the ANOVA, lack-of-fit test, three R^2 statistics, and polynomial model for shoots > 2 mm are presented in Table-4 and a contour plot of the relationship of BA and mT to the number of shoots > 2 mm produced over the design space in Figure-6. A quadratic BA:*m*T mixture x quadratic growth regulator amount polynomial model was selected as the "best" representation of the number of shoots > 2 mm across the design space. The data were transformed as per a Box Cox plot analysis using a base 10 log data transformation (log10 (number of shoots > 2 mm + 0.35)) to stabilize the variance. The resulting model was highly significant ($p < 0.0001$). Residual analyses and diagnostic plots were within acceptable limits. The lack-of-fit test was not significant ($p = 0.4078$) and the R^2, R^2_{adj} and R^2_{pred} statistics were 0.92, 0.88, and 0.78, respectively. The ANOVA contained three significant terms - BA*concentration, BA*mT*concentration, and

BA*mT*concentration^2. No BA: mT proportional effects were detected. The region of the design space that resulted in the greatest number of shoots > 2 mm was at low levels (< 13.35 µM) of BA.

Table-1. Mixture-amount treatment points and data for the three responses measured for Hamlin sweet orange. Experiment is a two-component mixture of BA and mT crossed with the quantitative factor total cytokinin concentration. The mixture components are listed as proportions. For example, treatment point #7 included 25 µM BA + 25 µM mT for a total cytokinin concentration of 50 µM. The measured response at each treatment design point represents the mean of six duplicate plates.

Treatment design points	Mixture components		Factor	Measured responses		
	BA	*m*T	[Cytokinin] µM	Overall quality[a]	# Explants w/ shoots/buds	# Shoots > 2 mm
1	0.50	0.5	1	1.5	14	8
2	1	0	13.25	2.5	18	35
3	1	0	50	1	7	3
4	0	1	25.50	2	11	19
5	0.25	0.75	37.75	1.25	9	12
6	0	1	1	2	16	10
7	0.5	0.5	50	1	4	0
8	0	1	50	1.25	4	9
9	0.5	0.5	25.50	1.5	11	12
10	1	0	37.50	1.25	9	10
11	1	0	1	2.5	20	29
12	1	0	50	1	5	6
13	0.25	0.75	13.25	2.25	17	25
14	0	1	37.50	1.5	7	9
15	0.5	0.5	50	1	6	0
16	1	0	25.50	1.5	11	8
17	0.75	0.25	13.25	2.25	18	27
18	0	1	13.25	2	15	11
19	1	0	1	2.75	17	31
20	0.75	0.25	37.75	1.25	6	7
21	0	1	50	1.5	12	17
22	0	1	1	2	14	4

[a] Assessed using the gestalt rating of Niedz *et al*., (2007) that utilized a scale of 1 to 3 where scores of 1, 2, or 3 corresponded to poor, intermediate, and good overall quality.

Table-2. ANOVA, regression coefficients, and summary statistics for explant quality (i.e., gestalt).

Source	F Value	p-values	Regression coefficients[b]
Model[a]	52.25	< 0.0001	
Linear Mixture	12.46	0.003	
BA			+ 0.32
meta-topolin			+ 0.24
BA * *m*T	16.73	0.001	+ 0.64
BA * Concentration	182.23	< 0.0001	+ 0.45
*m*T * Concentration	26.01	0.0001	+ 0.17
BA * Concentration^2	17.00	0.0009	+ 0.26
*m*T * Concentration^2	6.58	0.0215	+ 0.16
Lack of Fit	p = 0.2878		
R^2	0.95		
R^2 adjusted	0.94		
R^2 predicted	0.90		
Model type[c]	Reduced quadratic mixture x quadratic amount		
Transformation[d]	Power (lambda = -2.11)		

[a] $(\text{Gestalt})^{-2.11}$ = 0.13135*BA + 0.23666*mT + 0.63624*BA*mT - 3.35592E-003*BA*Concentration - 6.58252E003*mT*Concentration + 4.26328E-004*BA*Concentration^2 + 2.65272E-004*mT *Concentration^2
[b] 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.
[c] Model reduction by backward elimination.
[d]Determined by a Box Cox plot analysis.

Table-3. ANOVA, regression coefficients, and summary statistics for the number of explants with buds and/or shoots.

Source	F value	p-values	Regression coefficients[b]
Model[a]	25.58	< 0.0001	
Linear Mixture	0.58	0.4552	
BA			+ 12.10
meta-topolin			+ 11.56
BA * Concentration	46.87	< 0.0001	- 6.92
*m*T * Concentration	17.55	0.0006	- 10.71
Lack of Fit	p = 0.8355		
R^2	0.81		
R^2 adjusted	0.78		
R^2 predicted	0.69		
Model type[c]	linear mixture x linear amount		
Transformation[d]	None		

[a] # explants w/ shoots/buds = 19.39595*BA + 15.64467*mT - 0.28515*BA*Concentration - 0.17450*mT*Concentration
[b] 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.
[c] Model reduction by backward elimination.
[d] Data transformation requirements were determined by a Box Cox plot analysis.

Table-4. ANOVA, regression coefficients, and summary statistics for the number of shoots > 2 mm.

Source	**F value**	**p-values**	**Regression coefficients[b]**
Model[a]	19.83	< 0.0001	
Linear Mixture	0.49	0.4943	
BA			+ 1.15
meta-topolin			+ 1.19
BA * *m*T	1.18	0.2964	+ 0.71
BA * Concentration	23.53	0.0003	- 0.42
*m*T * Concentration	1.83	0.1994	+ 0.12
BA * *m*T * Concentration	18.18	0.0009	- 2.08
BA * Concentration^2	0.12	0.7391	- 0.056
*m*T * Concentration^2	1.95	0.1858	- 0.23
BA * *m*T * Concentration^2	18.43	0.0009	- 3.79
Lack of Fit	p = 0.4078		
R^2	0.92		
R^2 adjusted	0.88		
R^2 predicted	0.78		
Model type[c]	quadratic mixture x quadratic amount		
Transformation[d]	Log10 (# shoots > 2 mm + 0.35)		

[a] Log10(# shoots > 2 mm + 0.35) = 1.52444*BA + 0.81727*mT - 1.23118*BA*mT - 0.012429*BA*Concentration + 0.024219*mT*Concentration + 0.23716*BA*mT*Concentration - 9.28232E-005*BA*Concentration^2 - 3.81083E-004*mT*Concentration^2 - 6.31370E-003*BA*mT*Concentration^2

[b] 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.

[c] Model reduction by backward elimination.

[d] Data transformation requirements were determined by a Box Cox plot analysis.

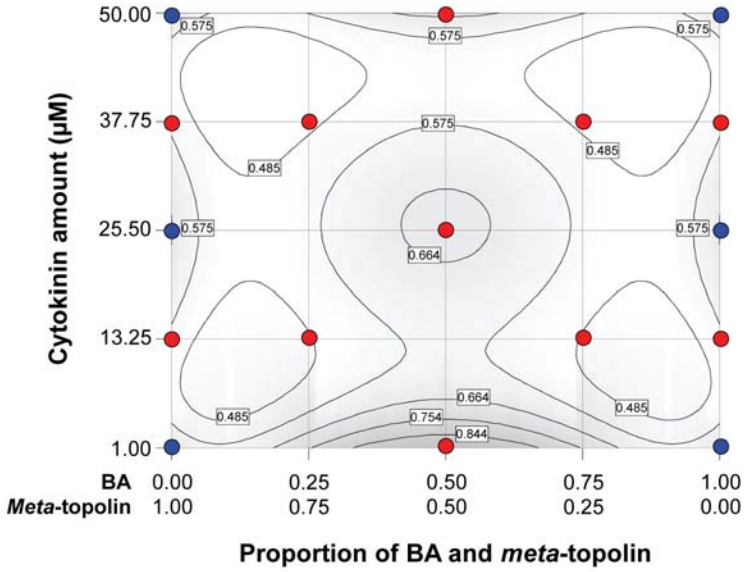

Figure-1. Experimental design space with treatment points. BA:*m*T mixture crossed with total cytokinin concentration design space with contours of the standard error of prediction. Explant response at the blue treatment points are shown in Figure-4.

Figure-2. Samples of explants used for gestalt ratings to evaluate the quality response. Explants were scored with a 3-level "gestalt rating", i.e. A) 1 = no response, B) 2 = neither 1 nor 3, C) 3 = strong response.

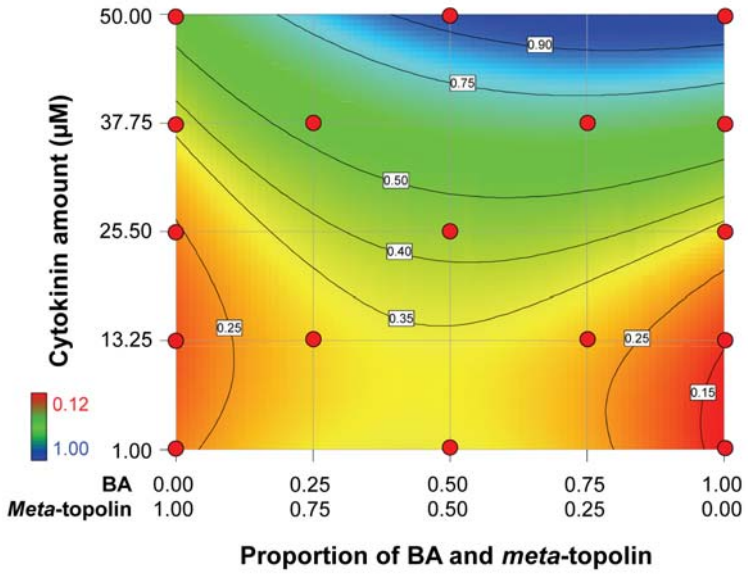

Figure-3. "Quality" gestalt response contour plot. The design space is a BA: mT mixture crossed with total cytokinin concentration.

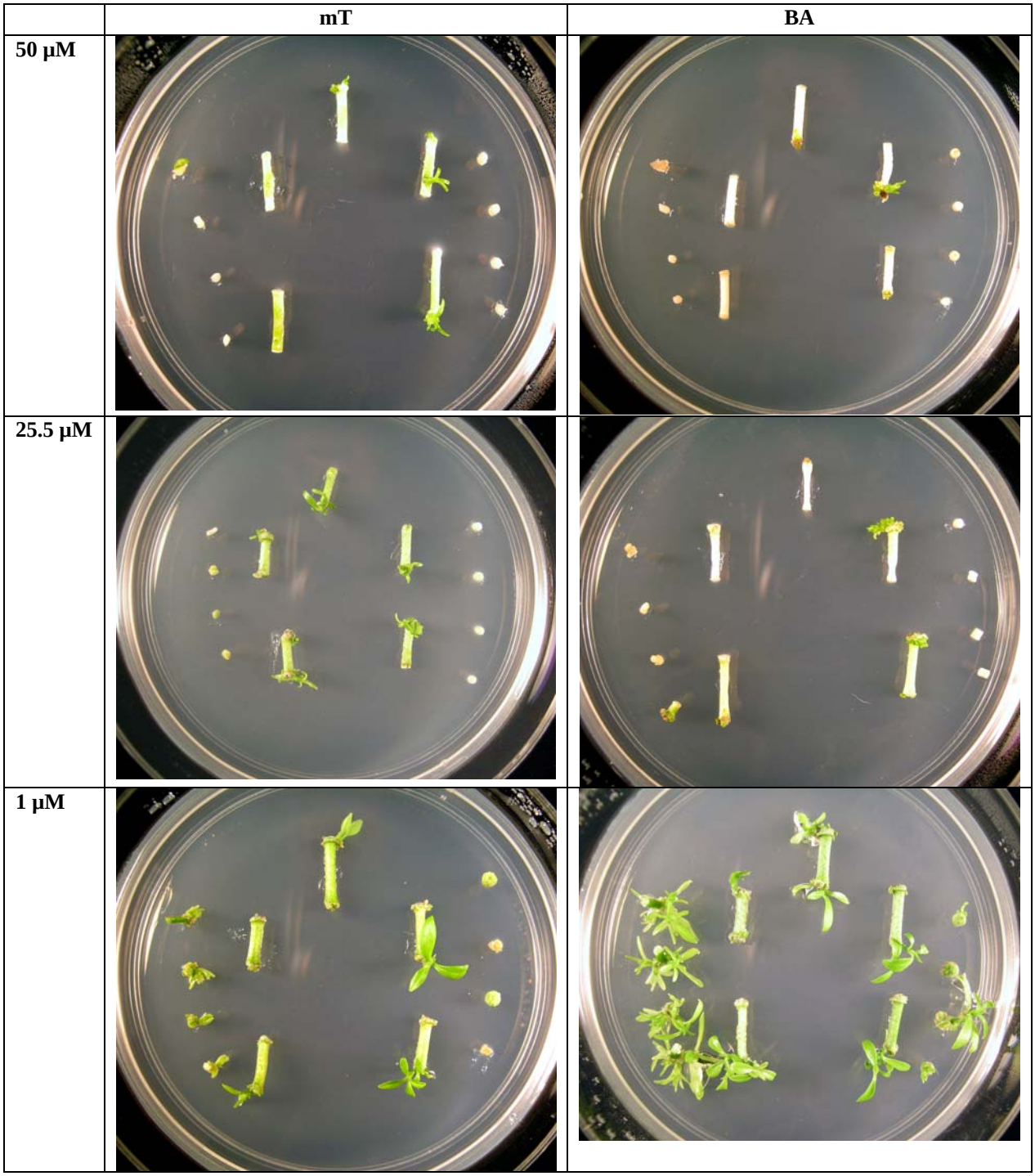

Figure-4. Explant response at six locations in the design space. Explant response at the six blue treatment points indicated in Figure-1. Points were chosen to provide representative examples of the observed range of responses.

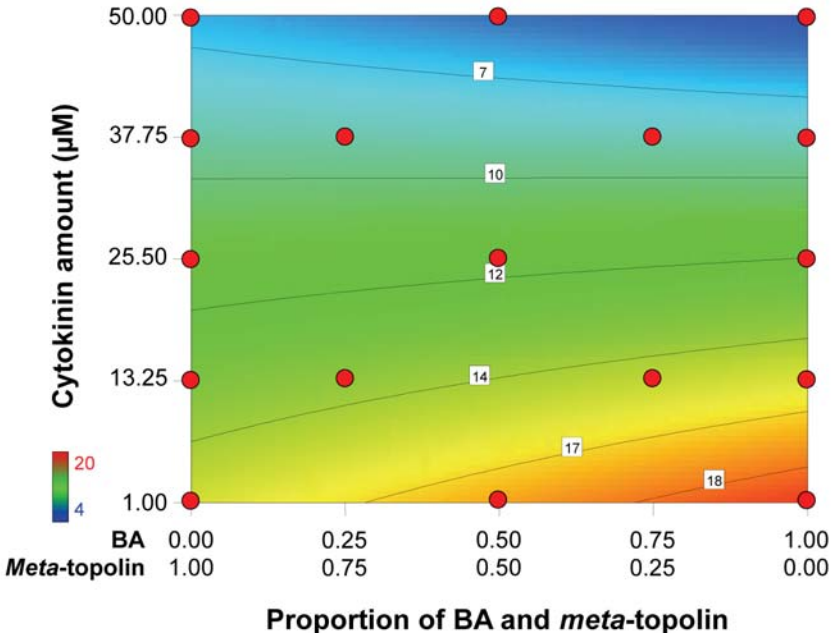

Figure-5. Contour plot of the number of explants with buds and/or shoots. The design space is a BA: mT mixture crossed with total cytokinin concentration.

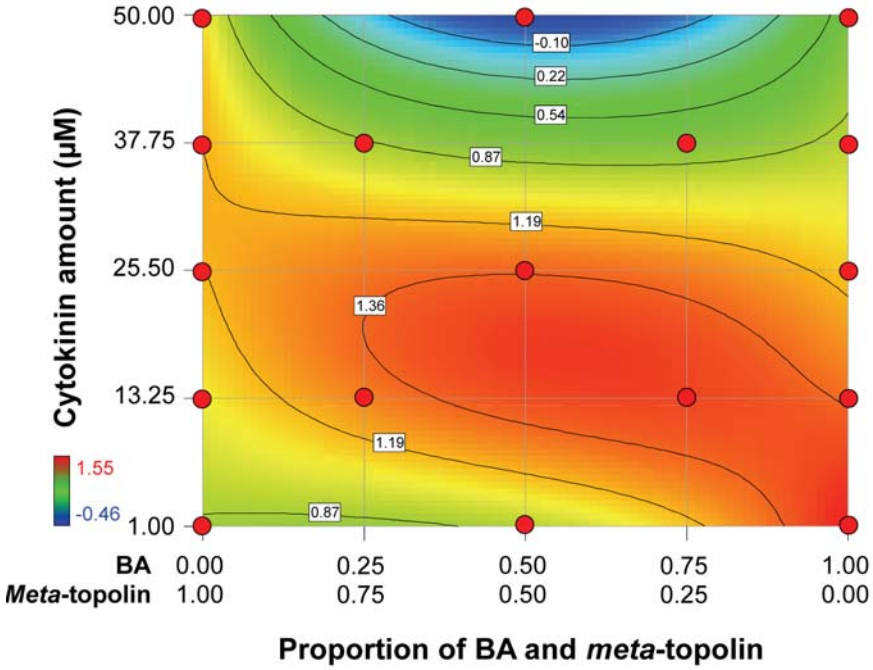

Figure-6. Contour plot of the number of shoots > 2 mm. The design space is a BA: mT mixture crossed with total cytokinin concentration.

DISCUSSIONS

To determine the effects of both proportion and concentration of BA and *m*T on shoot regeneration of 'Hamlin' sweet orange a mixture-amount experiment was designed. Unlike factorial designs, mixture-amount designs do not confound the effects of proportion and concentration. By separating these effects, we were able to answer the following questions.

a) *Are mixtures of BA and mT better or worse than either cytokinin alone?* Proportion-dependent effects were either not detected (number of explants w/ shoots/buds and number shoots > 2 mm) or were statistically significant but had minimal effect (e.g., overall quality). Proportion-dependent effects are evidenced by a non-linear response across the proportionality axis (x-axis orientation), i.e., the effects are not solely driven by concentration. We concluded that BA and

*m*T exhibit no or minimal synergistic or antagonistic effects on quality of shoot regeneration, number of explants that produce shoots, and number of shoots > 2 mm.

b) *Is the total amount of cytokinin added to the medium important?* Yes. The BA*concentration and *m*T*concentration terms were prominent in each of the three modeled responses, though the BA*concentration term was the most important (as determined by F-values). This is because *m*T, though less effective than BA, exhibited positive effects over a wider concentration range; whereas, the effect of BA became increasingly negative as the concentration increased.

c) *Does the BA: mT proportions interact with concentration?* The BA**m*T* concentration term was not significant for overall quality or number of explants with shoots/buds, but was significant (along with the related BA**m*T*concentration ^{2} term) for the number of shoots > 2 mm. The concentration of BA was the single largest determinant of the magnitude of each response.

If we had examined cytokinin effects individually, i.e., with one-factor-at-a-time (OFAT) experiments, then we would have been able to answer only question #2. Questions #1 and #3 both require a mixture-amount experimental design. This may be of particular importance in follow-up experiments that include other plant growth regulators, such as auxins, in combination with a cytokinin or set of cytokinins where synergistic interactions are generally the rule rather than the exception. The effects of BA and *m*T on the three measured responses was similar across the design space and showed that overall quality, number of explants with shoots/buds, and the number of shoots > 2 mm are closely related. Overall quality or "gestalt" measure (Niedz *et al.*, 2007) is an important response to include in a tissue culture study as it is 1) quick and easy to measure, 2) is easily analyzed by standard ANOVA, 3) captures overall quality utilizing the expertise of the tissue culturist, and 4) is inherently multivariate and will detect important differences, if they exist, that may not be captured by single metric type responses (i.e., something the tissue culturist is seeing that is not adequately measured with any single metric response). Some logical follow-up studies to the research presented here might include the effect of BA and *m*T on rooting, *ex vitro* acclimatization, and somaclonal variation since differential effects have been reported in other species (Bairu *et al.*, 2007; Bairu *et al*., 2008, Bairu *et al*., 2009), and including other plant growth regulators to identify blends (proportion and concentration) to optimize shoot regeneration and growth.

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