AMARANTH TISSUE CULTURE FOR THE PRODUCTION OF NATURAL FOOD COLORANTS

By

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In finishing, I would like to dedicate this thesis to a very dear friend I lost in October of 1992. We will miss you little buddy, and we'll meet again, I promise.

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CHAPTER I

INTRODUCTION

Visual appearance is an important quality factor of food. Color is an important component of visual appearance (Clydesdale, 1991). Color is perceived by humans based on responses of three different receptors (red, green, and blue) in the human eye (Francis and Clydesdale, 1975; Gnanasekharan et al., 1992) and is inherently subjective (Gnanasekharan et al., 1992). This subjectivity has led to the use of colorimeters to measure the tristimulus L* (a measure of the lightness or darkness), a* (a measure of the redness or greenness), and b* (a measure of the blueness or yellowness) values (Francis, 1980; Shewfelt et al., 1988; Clydesdale, 1991; Gnanasekharan et al., 1992; Trail et al., 1992). Color, as more closely perceived by humans, has been indicated by the conversion of these readings to hue angle ($\tan^{-1}$ b/a) and chroma $(a^2 + b^2)^{½}$ (Shewfelt et al., 1988; Gnanasekharan et al., 1992; Trail et al., 1992).

Color may be inherent in food or added to food. Color additives may be in the form of natural or artificial dyes. Questions regarding the safety of some artificial dyes in food have led researchers to find ways of substituting these dyes with natural pigments (Böhm and Rink, 1988). These

natural pigments can be obtained through plant tissue culture techniques (Ilker, 1987). Plant tissue culture has been used for *in vitro* propagation of rare and valuable crops and biochemicals (Knorr et al., 1990). Sanders et al. (1993) suggest priorities for future research including development of color from plants using biotechnology. The ever increasing list of products produced from plant tissue culture include food flavors such as vanilla, medicines, caffeine and nicotine. This list also contains natural pigments such as anthocyanins and betalains, both of which are water-soluble, vacuolar pigments. Betalains are naturally occurring red pigments produced primarily by sources in the order Centrospermae (Böhm and Rink, 1988). This order contains the families Amaranthaceae (*Amaranthus sp.*) and Chenopodiaceae (*Beta sp.*).

The red beet (*Beta vulgaris*) is an important source of betalain pigment (Böhm and Rink, 1988) and the extracted betalains are currently being used as food colorants in the food industry (Kang et al., 1992). However, cell cultures of *Beta vulgaris* have a smell and taste typical of red beet (Böhm and Rink, 1988). Also, betalain-decolorizing enzymes are present in cell cultures of *Beta vulgaris* and *Amaranthus tricolor* seedlings (Böhm and Rink, 1988). Therefore, it is necessary to purify the betalain extracts of these cell cultures in order to remove the smell, taste and enzymes, a process which complicates the use and stability of betalains as a natural food dye (Böhm and Rink, 1988).

Amaranth (*Amaranthus sp.*) has been researched for production of betalains; however, the research has centered around production in seedlings (French et al., 1973; Kochhar, et al., 1981; Elliott, 1983). The work that has been done concerning production of betalains from calli show production of pigment occurs only under certain conditions (Böhm and Rink, 1988). *Amaranthus caudatus* and *Amaranthus tricolor* have been researched for the production of betalains in seedlings propagated through plant tissue culture. Betalain pigment was produced by *Amaranthus caudatus* in a static culture on a modified B5 medium (Constabel and Nassif-Makki, 1971), and Davis and Guenzi (1988) noticed pigmentation in calli initiated from seeds of *Amaranthus hypochondriachus* in a static culture on a MS (Murashige and Skoog, 1962) medium. Kang et al. (1992) found betalain production the greatest with 6% sucrose, 200 mg/L phosphate and concentrations of nitrogen in the range of 0.055 to 0.082 M. Kang et al. (1992) also found that calli induced by 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) were friable and brittle. Davis and Guenzi (1988) found that production of betalain was enhanced by a combination of 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.5 μM BAP.

Betalain production is stimulated by light (French et al., 1973). Several studies indicate that the biosynthesis of betacyanins is under phytochrome control (Köhler, 1972; French et al., 1973). Monochromatic green light was

reported to promote much greater betalain production than other monochromatic light (red and blue) in red beets (Kang et al., 1992). Since *Amaranthus* contains a significant amount of betalains in the mature plants and organs (French et al., 1973), an efficient method of producing the pigment through plant tissue culture can lead to a cost effective use in the food industry.

The objectives of this study are: 1) to identify an efficient plant tissue culture method for production of betalain from *Amaranthus hypochondriachus*, 2) to determine the effect of 2,4-D concentration on callus formation and growth of *Amaranthus hypochondriachus*, 3) to determine the effects of sucrose, phosphate, BAP and 2,4-D concentrations in medium on growth and betalain production of *Amaranthus hypochondriachus*, and 4) to determine the effect of light wavelength on growth and betalain production of *Amaranthus hypochondriachus*.

CHAPTER II

MATERIALS AND METHODS

Explant Source

Seeds of *Amaranthus hypochondriachus* were obtained from L. Weber of the Rodale Research Center, Emmaus, Pennsylvania. *A. hypochondriachus* (cultivar 1023) was chosen because of its inclination to show pigmentation (Davis and Guenzi, 1988). Seeds were surface sterilized by washing in 70% ethanol for 5 minutes, then washing in 20% Clorox solution with t-octylphenoxypolyethoxyethanol (50µL/500 ml) for 20 minutes. Seeds were rinsed with sterile water for 5 minutes after each wash (Pierik, 1987).

Sample Evaluation

Evaluation of Growth

Callus fresh weights were recorded at day zero and at the beginning of each subsequent transfer period. This was accomplished by weighing each callus as it was transferred to a new plate. The relative growth rates were calculated from the fresh weight values using the following equation (Singer and McDaniel, 1986):

Relative Growth Rate: (ln[end wt] - ln[begin wt])/days

Evaluation of Color

Colorimetric measurements were recorded for each transfer period (approximately every 2 to 4 weeks) using a Minolta Chroma Meter series CR-200 after calibration with a white standard tile (L = 97.75, a = -0.58, b = 2.31). Differences in surface pigmentation of larger calli were compensated by reporting the average of 2 readings (Gnanasekharan et al., 1992). More than 2 readings were not necessary, as the calli were subcultured before they became too large. This non-destructive color determination was recorded under sterile conditions at day zero and at the beginning of each subsequent transfer period. The L*a*b* readings were used to compute the following derived functions (Gnanasekharan et al., 1992; Trail et al., 1992):

$$\text{Hue angle:} \quad \tan^{-1}(b/a)$$

$$\text{Chroma:} \quad (a^2 + b^2)^{1/2}$$

$$\text{Total color difference (DE):} \quad [(L-L_0)^2 + (a-a_0)^2 + (b-b_0)^2]^{1/2}$$

Where L_0, a_0, and b_0 represent the respective readings at day zero.

Statistical Analysis

Data from each experiment were subjected to analysis of variance (ANOVA) using the General Linear Model procedure (SAS, 1985).

Experiment I: Callus Initiation.

MS (Murashige and Skoog, 1962) medium was used (see appendix). Two 2,4-D concentrations (5.7 and 22.6 µM) were evaluated for the promotion of callus formation. Media were solidified with 2.5 g L^{-1} Phytagel (Sigma Chem. Co., St. Louis, MO 63178) and sterilized by autoclaving. Medium was poured into sterile disposable petri dishes. Twenty surface-sterilized seeds were inoculated on each plate. Forty plates were used for each treatment. Tissue culture were grown in a controlled environment room at 25°C in the dark. Callus initiation was evaluated for each medium and relative growth rates were calculated for callus as described above. The medium which induced the best initiation and growth of callus was determined and used for experiment II.

Experiment II: Media Manipulation for Growth Rate and Betalain Production.

The sucrose, phosphate, BAP and 2,4-D concentrations were manipulated to test for optimum growth and betalain production. MS basal medium was used for all treatments. Treatments consisted of combinations of sucrose (55.56 mM, 111.11 mM, 166.67 mM), phosphate (1.25 mM, 2.5 mM), BAP (0 µM, 2.5 µM) and 2,4-D (10 µM, 15 µM) (Table 1).

TABLE 1

ADDITIVES APPLIED TO MS MEDIA FOR IN VITRO CULTURING OF AMARANTHUS HYPOCHONDRIACHUS

Treatment	Sucrose (mM)	Phosphate (mM)	BAP* (μM)	2,4-D** (μM)
0000	55.56	1.25	0.0	10
1000	111.11	1.25	0.0	10
2000	166.67	1.25	0.0	10
0100	55.56	2.50	0.0	10
1100	111.11	2.50	0.0	10
2100	166.67	2.50	0.0	10
0010	55.56	1.25	2.5	10
1010	111.11	1.25	2.5	10
2010	166.67	1.25	2.5	10
0110	55.56	2.50	2.5	10
1110	111.11	2.50	2.5	10
2110	166.67	2.50	2.5	10
0001	55.56	1.25	0.0	15
1001	111.11	1.25	0.0	15
2001	166.67	1.25	0.0	15
0101	55.56	2.50	0.0	15
1101	111.11	2.50	0.0	15
2101	166.67	2.50	0.0	15
0011	55.56	1.25	2.5	15
1011	111.11	1.25	2.5	15
2011	166.67	1.25	2.5	15
0111	55.56	2.50	2.5	15
1111	111.11	2.50	2.5	15
2111	166.67	2.50	2.5	15

*BAP - 6-benzylaminopurine

**2,4-D - 2,4-dichlorophenoxyacetic acid

The experimental design consisted of 4 replicates (petri plates) per treatment. Four calli were evaluated per replicate. Media were sterilized and aseptically transferred into sterile petri dishes (as described above). Calli from medium exhibiting the best initiation and growth from Experiment I were included in this experiment. The calli were evaluated for relative growth rates and pigmentation as described above.

Experiment III: Light Manipulation for Growth Rate And Betalain Production.

A photoperiodic regime of 16 hours of the light treatment and 8 hours of darkness was maintained. The light treatments consisted of red monochromatic light, green monochromatic light, white light and dark (control). Red monochromatic light was achieved by using red-colored film which allowed peak transmission at a wavelength of 650 nm. The green monochromatic light was achieved by using green-colored film which allowed peak transmission at a wavelength of 520 nm.

Tissue cultures were grown in a controlled environment room in which the temperature was maintained at 25°C. The experiment was carried out with five replicates for each of the light treatments. Four calli were evaluated per replicate. The medium exhibiting the best callus growth and pigment production from experiment II was utilized in this

experiment. Relative growth rates and pigmentation were evaluated for each callus as described above.

CHAPTER III

RESULTS

Callus Initiation

The callus initiation on MS medium with a 2,4-D concentration of 5.7 µM was 90.37%. Initiation on MS medium with a 2,4-D concentration of 22.6 µM was 88.03%. Calli on 5.7 µM medium exhibited a relative growth rate of 0.020, whereas the relative growth rate of calli on 22.6 µM medium were slightly lower (0.018). However, calli grown on the 5.7 µM medium produced root tissue in abundance, unlike the calli grown on medium containing 22.6 µM 2,4-D. The 2,4-D concentration of 5.7 µM was increased to 10.0 µM in order to inhibit root growth. The 2,4-D concentration of 22.6 µM was decreased to 10 µM to increase callus growth rate. The calli of both 5.7 µM and 22.6 µM exhibited increased relative growth rates when transferred to the new 2,4-D concentration (0.08 and 0.04, respectively). The higher growth rate of 5.7 µM calli after transfer to new 2,4-D concentration was due to the growth of root tissue, which could not be eliminated. Due to the abundance of root growth in these calli, the calli from 22.6 µM were chosen

for further experimentation. The 22.6 μM exhibited no root growth and the calli were compact and clear.

Media Manipulation

Evaluation of Growth

There was a difference ($P \leq 0.0001$) for relative growth rates between media treatments (Table 2). At transfer period 1, treatments 1010 and 1110 exhibited highest relative growth rates (Figure 1). By transfer period 2, the high relative growth rates shifted to treatments 1100, 1111 and 2111 (Figure 1). Treatments 1100, 1011 and 1111 possessed the higher relative growth rates by transfer period 3 (Figure 1).

Since no sub-culturing occurred through transfer period 2, the overall relative growth rates were calculated for this time and are presented in Figure 2. Through period 2, treatments 1010, 1110 and 1111 exhibited the highest overall relative growth rates (Figure 2). The growth rates of these treatments (1010, 1110 and 1111) were not different from each other ($P \geq 0.05$).

Treatments exhibiting the highest overall relative growth rates (through period 2) have in common a sucrose content of 111.11 mM and a BAP concentration of 2.5 μM (Table 1). Among the lowest overall relative growth rates (through period 2) are treatments 2000, 2100, 2010, 2001,

TABLE 2

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN RELATIVE GROWTH RATES OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	0.042	0.030	0.015	0.005
0100	55.56	2.50	0.00	10.00	0.045	0.042	0.020	0.009
0010	55.56	1.25	2.50	10.00	0.056	0.023	1.021	0.007
0110	55.56	2.50	2.50	10.00	0.052	0.032	0.041	0.007
0001	55.56	1.25	0.00	15.00	0.050	0.029	0.026	0.005
0101	55.56	2.50	0.00	15.00	0.040	0.037	0.043	0.005
0011	55.56	1.25	2.50	15.00	0.048	0.032	0.019	0.010
0111	55.56	2.50	2.50	15.00	0.044	0.039	0.014	0.005
1000	111.11	1.25	0.00	10.00	0.034	0.045	0.037	0.006
1100	111.11	2.50	0.00	10.00	0.036	0.049	0.047	0.008
1010	111.11	1.25	2.50	10.00	0.063	0.035	0.043	0.006
1110	111.11	2.50	2.50	10.00	0.061	0.043	0.041	0.009
1001	111.11	1.25	0.00	15.00	0.035	0.039	0.035	0.009
1101	111.11	2.50	0.00	15.00	0.027	0.047	0.029	0.007
1011	111.11	1.25	2.50	15.00	0.041	0.047	0.050	0.008
1111	111.11	2.50	2.50	15.00	0.046	0.051	0.050	0.007
2000	166.67	1.25	0.00	10.00	0.023	0.028	0.025	0.004
2100	166.67	2.50	0.00	10.00	0.023	0.037	0.024	0.006
2010	166.67	1.25	2.50	10.00	0.026	0.044	0.032	0.007
2110	166.67	2.50	2.50	10.00	0.026	0.049	0.029	0.006
2001	166.67	1.25	0.00	15.00	0.017	0.025	0.016	0.003
2101	166.67	2.50	0.00	15.00	0.019	0.030	0.022	0.005
2011	166.67	1.25	2.50	15.00	0.027	0.039	0.029	0.007
2111	166.67	2.50	2.50	15.00	0.025	0.051	0.028	0.004
				LSD **	0.008	0.006	0.008	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

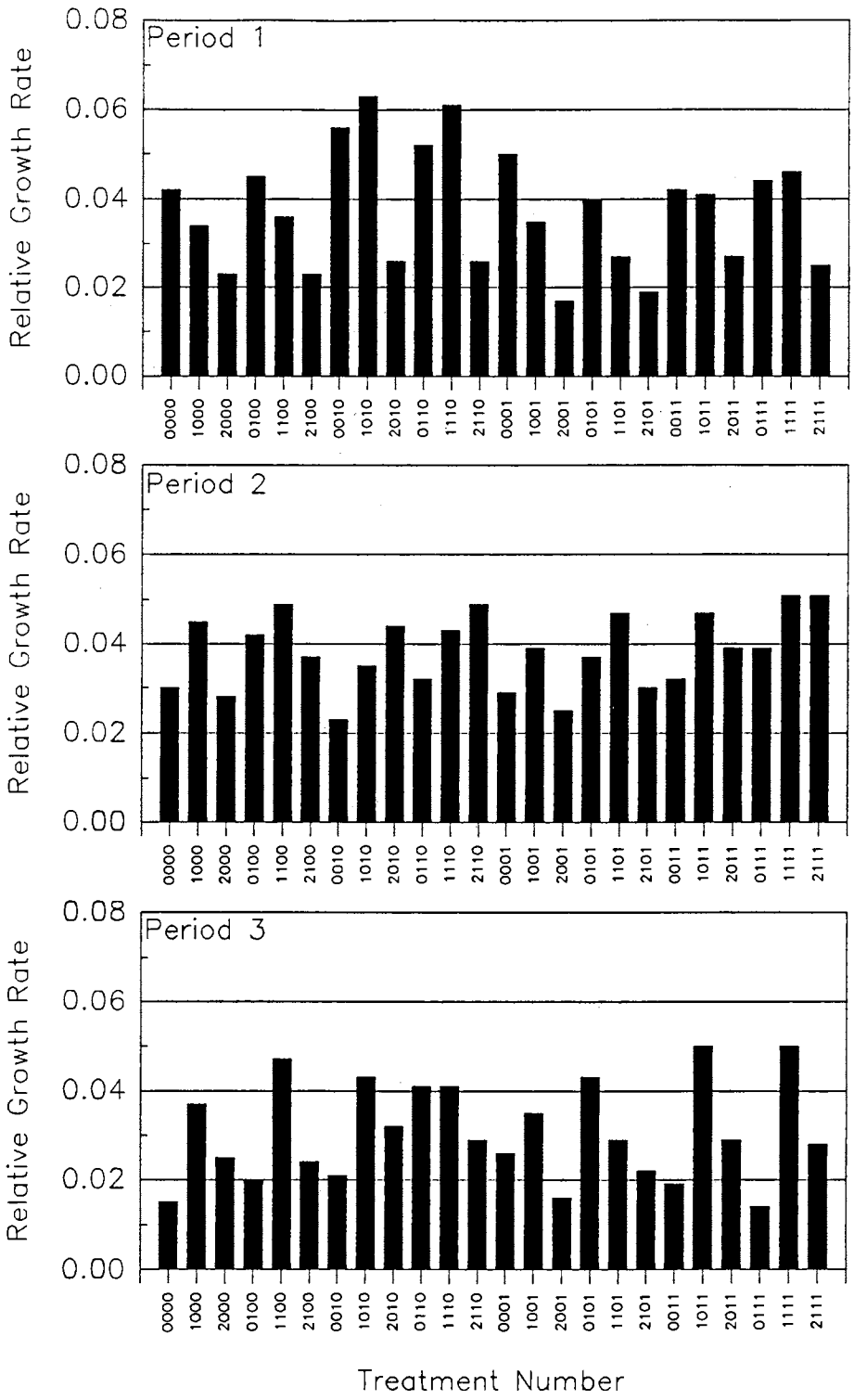

Figure 1. Relative growth rates of *Amaranthus hypochondriachus* calli as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 µM, 1 = 2.5 µM), and 2,4-D (0 = 10.0 µM, 1 = 15.0 µM).

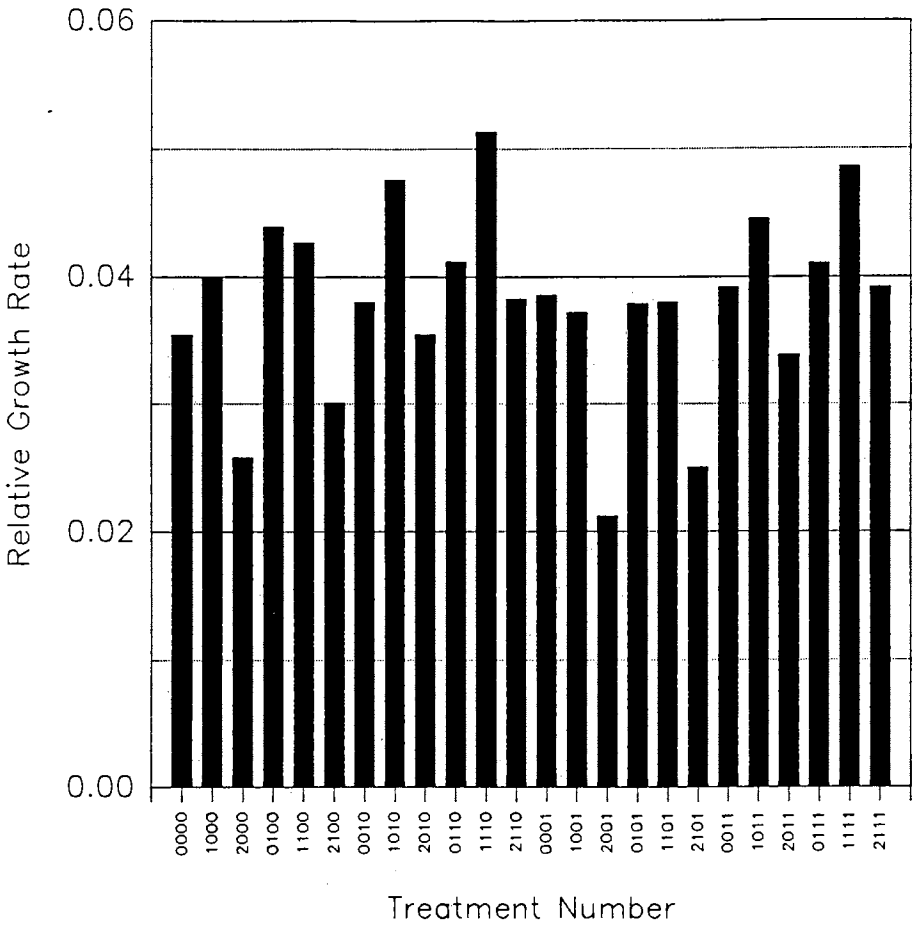

Figure 2. Overall relative growth rates of *Amaranthus hypochondriachus* calli through transfer period 2, as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 µM, 1 = 2.5 µM), and 2,4-D (0 = 10 µM, 1 = 15 µM).

2101 and 2011 (Figure 2). These treatments have in common 166.67 mM sucrose content.

The growth rates as influenced by each component concentration for each period are presented in Table 3. The concentration of sucrose (55.56 mM, 111.11 mM and 166.67 mM) influenced ($P \leq 0.0001$) the growth rates in each of the three periods. At the first transfer period, treatments containing 55.56 mM sucrose showed the highest relative growth rates followed by treatments containing 111.11 mM sucrose, which was then followed by treatments containing 166.67 mM sucrose. With the exception of the first period, treatments with 111.11 mM sucrose exhibited the highest relative growth rates (Table 3). By period 3, the growth rates of treatments with 111.11 mM sucrose were significantly higher than the treatments containing sucrose levels of 55.56 mM and 166.67 mM, while the growth rates of treatments containing 55.56 mM and 166.67 mM were not different from each other ($P \geq 0.05$).

The concentration of phosphate (1.25 mM and 2.50 mM) had no significant effect on the relative growth rate for the first period, while higher phosphate increased the relative growth rate in the second ($P \leq 0.0001$) and third ($P \leq 0.05$) periods (Table 3). In both period 2 and period 3, the highest relative growth rates were obtained in media which contained 2.50 mM phosphate. Of the three treatments exhibiting the highest overall relative growth rates (Figure 2) through period 2 (1010, 1110 and 1111), only treatments

TABLE 3

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) CONCENTRATION ON THE MEAN RELATIVE GROWTH RATES OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Component	Concentration	Transfer Period			LSD *
		1	2	3	
Sugar	55.56	0.047	0.033	0.023	0.003
(mM)	111.11	0.043	0.044	0.042	0.003
	166.67	0.023	0.038	0.026	0.002
	LSD *	0.003	0.003	0.003	
Phosphate	1.25	0.038	0.035	0.029	0.003
(mM)	2.50	0.037	0.042	0.032	0.003
	LSD *	0.003	0.002	0.003	
BAP	0.00	0.033	0.036	0.028	0.002
(µM)	2.50	0.043	0.040	0.033	0.003
	LSD *	0.003	0.002	0.003	
2,4-D	10.00	0.040	0.038	0.031	0.003
(µM)	15.00	0.035	0.039	0.030	0.003
	LSD *	0.003	0.002	0.003	

* LSD - Least Significant Difference (0.05).

1110 and 1111 contained the higher phosphate concentration (2.50 mM).

Relative growth rates were higher ($P \leq 0.0001$) when BAP was added at 2.5 µM in each of the three periods (Table 3). All three treatments which exhibited the highest overall relative growth rates (through period 2) contained the higher BAP concentration (2.5 µM).

The lower concentration of 2,4-D (10 µM vs. 15 µM) increased ($P \leq 0.0001$) the relative growth rate for period 1 only (Table 3). This was consistent with results from the callus initiation experiment. During callus initiation, the relative growth rate increased as the concentration of 2,4-D was lowered from 22.6 µM to 10.0 µM.

A summary of ANOVA results for relative growth rates throughout the entire experiment was developed for each component and their interactions (Table 4). The four-way interaction (SxPxBxD) was not significant ($P \geq 0.05$) (Table 4). Sucrose and BAP exhibited a highly significant ($P \leq 0.0001$) two-way interaction (SxB) and three-way interaction with the addition of 2,4-D (SxBxD) (Table 4). Sucrose and phosphate do not exhibit a significant ($P \geq 0.05$) two-way interaction (SxP); however, when 2,4-D is added, the resulting three-way interaction (SxPxD) is significant ($P \leq 0.05$) (Table 4).

TABLE 4

THE EFFECTS OF SUCROSE (S), PHOSPHATE (P), 6-BENZYLAMINOPURINE (B), AND 2,4-DICHLOROPHENOXYACETIC ACID (D) AND THEIR INTERACTIONS ON THE MEAN RELATIVE GROWTH RATES OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Component	Statistical Significance
S	***
P	***
B	***
D	**
SxP	NS
SxB	***
SxD	NS
PxB	NS
PxD	NS
BxD	NS
SxPxB	NS
SxPxD	*
SxBxD	***
PxBxD	NS
SxPxBxD	NS

Significance of components and their interactions are indicated as: *** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$, NS - Not Significant.

Evaluation of Color

Values of tristimulus L*, a*, and b*, were measured and summaries of these results are presented in Table 5, Table 6, and Table 7, respectively. Stimulus L* measures the lightness or darkness of the callus. Higher 'L' values indicate a relatively lighter color than lower 'L' values. Within each of the four periods, the 'L' values were different ($P \leq 0.0001$) between the treatments. Each treatment exhibited a difference between periods significant at least at $P \leq 0.05$ (Table 5). Treatments 1110 and 0111 were the exception, with both exhibiting similar 'L' values for the final period and the initial period (Table 5). Treatment means of stimulus L* for the experiment are plotted in Figure 3.

Stimulus a* measures the redness ('a' > 0) or greenness ('a' < 0) of the callus. The initial period exhibited a difference ($P \leq 0.01$) between the treatments for the 'a' values. Each of the following three transfer periods also exhibited a difference ($P \leq 0.0001$) for these values. Each treatment exhibited a significant difference between periods of at least $P \leq 0.05$ (Table 6). The final 'a' values were higher ($P \leq 0.05$), indicating more red color than the initial 'a' values in 19 of these 23 treatments (Table 6). Treatment means of stimulus a* for the experiment are plotted in Figure 3. In each group of three treatments, varying only the levels of sucrose, treatments with media

TABLE 5

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN L* VALUE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 0	1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	28.56	34.96	33.29	34.80	2.44
0100	55.56	2.50	0.00	10.00	30.59	40.26	32.05	32.96	2.71
0010	55.56	1.25	2.50	10.00	30.60	37.00	33.04	35.25	2.52
0110	55.56	2.50	2.50	10.00	33.98	38.77	34.70	38.70	2.52
0001	55.56	1.25	0.00	15.00	31.13	37.18	35.07	39.39	2.63
0101	55.56	2.50	0.00	15.00	34.31	38.91	33.89	37.88	2.05
0011	55.56	1.25	2.50	15.00	27.75	34.42	31.68	32.97	2.68
0111	55.56	2.50	2.50	15.00	34.10	41.60	35.40	32.08	2.69
1000	111.11	1.25	0.00	10.00	30.51	36.31	32.88	36.87	2.45
1100	111.11	2.50	0.00	10.00	31.91	37.35	34.70	36.48	3.14
1010	111.11	1.25	2.50	10.00	33.18	37.92	31.14	36.73	2.93
1110	111.11	2.50	2.50	10.00	35.18	38.58	32.80	34.13	3.63
1001	111.11	1.25	0.00	15.00	30.69	35.74	34.92	36.59	3.09
1101	111.11	2.50	0.00	15.00	35.26	39.77	36.94	37.74	2.75
1011	111.11	1.25	2.50	15.00	30.11	36.28	30.98	34.36	2.76
1111	111.11	2.50	2.50	15.00	33.25	41.64	31.36	38.05	3.36
2000	166.67	1.25	0.00	10.00	32.35	37.05	32.84	39.47	2.42
2100	166.67	2.50	0.00	10.00	31.84	35.40	33.26	35.51	2.92
2010	166.67	1.25	2.50	10.00	30.48	33.14	32.35	37.81	2.94
2110	166.67	2.50	2.50	10.00	34.81	39.96	37.75	38.24	3.16
2001	166.67	1.25	0.00	15.00	29.24	33.37	35.70	37.30	2.89
2101	166.67	2.50	0.00	15.00	32.79	39.40	35.92	34.23	2.82
2011	166.67	1.25	2.50	15.00	31.78	32.46	34.16	36.21	2.59
2111	166.67	2.50	2.50	15.00	33.71	40.11	32.53	35.46	2.83
				LSD **	2.27	3.06	2.83	3.29	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

TABLE 6

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN a* VALUE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 0	1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	2.41	0.61	2.25	4.01	0.76
0100	55.56	2.50	0.00	10.00	2.20	2.11	2.44	3.77	1.02
0010	55.56	1.25	2.50	10.00	2.41	1.38	4.22	2.15	0.88
0110	55.56	2.50	2.50	10.00	1.75	1.66	3.37	2.24	0.75
0001	55.56	1.25	0.00	15.00	1.93	1.24	3.13	2.16	0.76
0101	55.56	2.50	0.00	15.00	1.49	1.54	2.82	1.80	0.96
0011	55.56	1.25	2.50	15.00	2.51	2.13	4.20	1.22	0.87
0111	55.56	2.50	2.50	15.00	1.91	1.52	3.76	4.91	0.79
1000	111.11	1.25	0.00	10.00	2.48	2.52	3.46	4.42	1.04
1100	111.11	2.50	0.00	10.00	1.73	3.17	2.69	4.82	1.40
1010	111.11	1.25	2.50	10.00	1.72	5.01	4.58	4.86	1.22
1110	111.11	2.50	2.50	10.00	1.41	4.40	4.46	3.85	1.24
1001	111.11	1.25	0.00	15.00	1.82	2.35	3.69	4.22	0.79
1101	111.11	2.50	0.00	15.00	1.53	2.90	2.98	2.81	1.02
1011	111.11	1.25	2.50	15.00	1.69	3.83	5.24	5.09	0.85
1111	111.11	2.50	2.50	15.00	1.97	2.62	4.69	5.40	1.63
2000	166.67	1.25	0.00	10.00	2.01	3.81	4.39	3.89	0.76
2100	166.67	2.50	0.00	10.00	1.60	2.97	5.00	6.17	1.11
2010	166.67	1.25	2.50	10.00	2.00	4.41	4.89	4.92	0.85
2110	166.67	2.50	2.50	10.00	1.60	3.71	4.45	5.70	0.95
2001	166.67	1.25	0.00	15.00	1.88	3.64	4.43	5.85	0.85
2101	166.67	2.50	0.00	15.00	1.83	3.86	4.57	4.99	0.99
2011	166.67	1.25	2.50	15.00	1.51	3.81	4.20	4.99	0.73
2111	166.67	2.50	2.50	15.00	1.40	4.44	4.82	5.51	0.78
				LSD **	0.62	1.17	0.84	1.18	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

TABLE 7

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN b* VALUE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 0	1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	13.68	16.29	16.70	17.75	1.68
0100	55.56	2.50	0.00	10.00	14.67	20.55	17.60	17.28	1.45
0010	55.56	1.25	2.50	10.00	14.58	19.27	18.22	16.88	1.76
0110	55.56	2.50	2.50	10.00	15.81	20.26	18.38	18.98	1.67
0001	55.56	1.25	0.00	15.00	14.52	18.27	17.62	19.62	1.80
0101	55.56	2.50	0.00	15.00	16.53	20.12	19.19	19.23	1.50
0011	55.56	1.25	2.50	15.00	13.68	18.02	16.79	15.62	1.80
0111	55.56	2.50	2.50	15.00	16.84	22.35	18.68	17.05	1.61
1000	111.11	1.25	0.00	10.00	14.79	19.93	19.83	20.09	1.44
1100	111.11	2.50	0.00	10.00	13.97	22.52	20.89	20.74	2.29
1010	111.11	1.25	2.50	10.00	14.80	23.11	20.38	22.52	1.97
1110	111.11	2.50	2.50	10.00	15.81	23.52	20.26	18.56	2.00
1001	111.11	1.25	0.00	15.00	13.95	19.04	19.36	19.67	2.17
1101	111.11	2.50	0.00	15.00	16.25	22.96	22.33	20.01	1.67
1011	111.11	1.25	2.50	15.00	12.96	21.90	19.60	21.67	2.09
1111	111.11	2.50	2.50	15.00	16.25	26.04	19.23	21.31	1.94
2000	166.67	1.25	0.00	10.00	14.10	19.02	18.28	20.14	1.62
2100	166.67	2.50	0.00	10.00	15.20	20.68	20.05	19.35	1.60
2010	166.67	1.25	2.50	10.00	13.94	18.60	19.69	20.81	1.94
2110	166.67	2.50	2.50	10.00	15.87	24.05	21.60	21.52	1.76
2001	166.67	1.25	0.00	15.00	13.53	17.18	19.17	19.74	1.51
2101	166.67	2.50	0.00	15.00	15.42	21.89	21.60	18.39	1.79
2011	166.67	1.25	2.50	15.00	14.28	17.84	18.60	19.26	1.57
2111	166.67	2.50	2.50	15.00	16.18	24.86	19.89	20.65	2.01
				LSD **	1.55	2.17	1.67	2.07	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

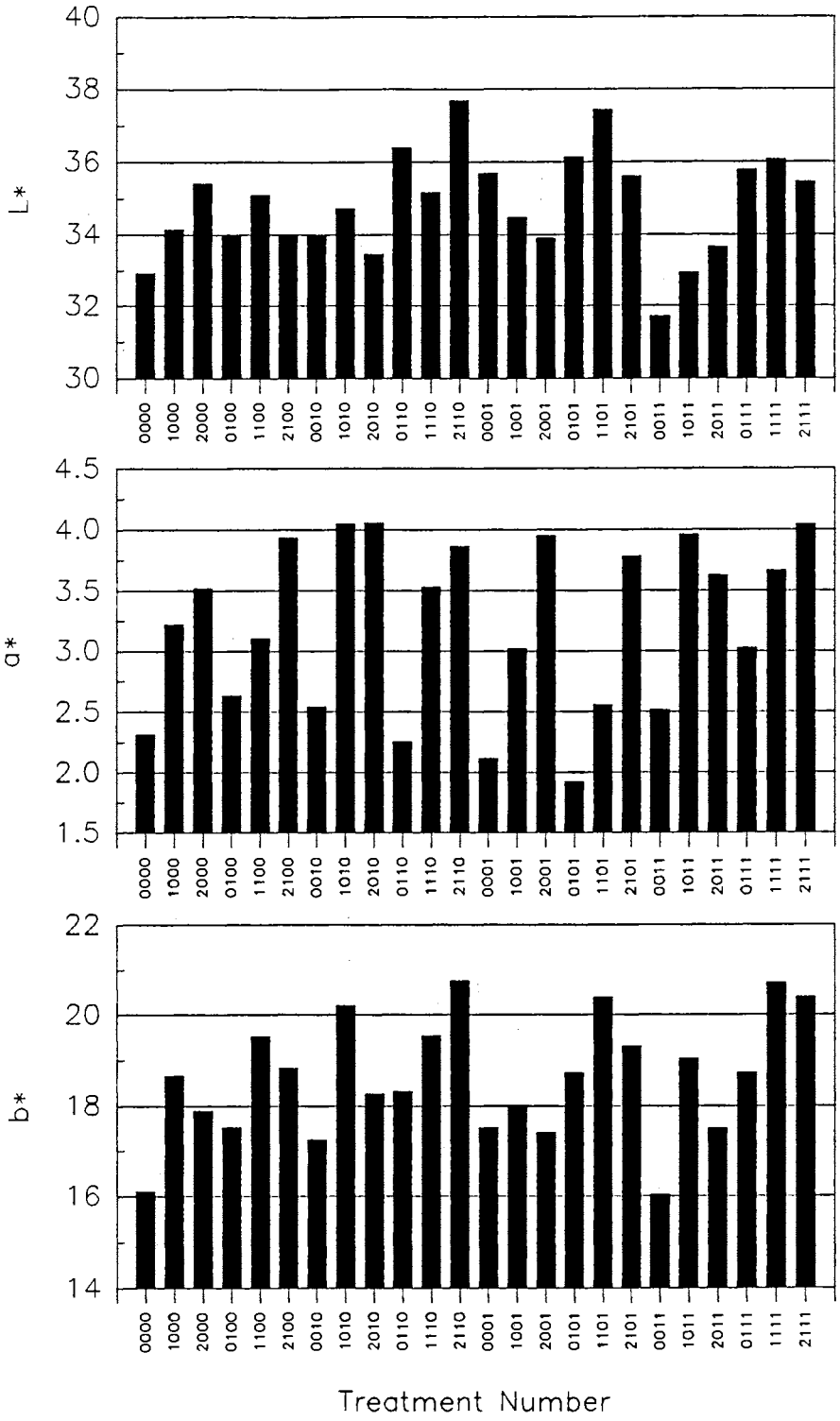

Figure 3. Treatment means for stimulus L*, a*, and b* values of *Amaranthus hypochondriachus* calli as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 µM, 1 = 2.5 µM), and 2,4-D (0 = 10 µM, 1 = 15 µM).

containing 166.67 mM sucrose exhibited more red color (higher stimulus a* values), with the exception of treatment 2011 (Figure 3). Differences between the 'a' value at the final transfer period and the 'a' value at the initial period for each treatment are plotted in Figure 4. Again, in each group of three treatments, varying only the levels of sucrose, treatments with media containing 166.67 mM sucrose exhibited a larger difference in the 'a' values, with the exception of treatments 2000 and 2010 (Figure 4).

Stimulus b* measures the yellowness ('b' > 0) or blueness ('b' < 0) of the callus. Within each of the four periods, the 'b' values were different ($P \leq 0.0001$) between the treatments. Each of the 24 treatments exhibited a difference ($P \leq 0.0001$) between periods (Table 7). In 23 of the 24 treatments (treatment 0111 is the exception), the final transfer period exhibited a significantly ($P \leq 0.05$) more yellow color (higher stimulus b* value than the initial stimulus b* value) (Table 7). Treatment means for stimulus b* for the experiment are plotted in Figure 3.

Total Color Difference. ΔE or the total color difference observed from the initial period was determined for each transfer period (Figure 5). The difference between treatments was significant ($P \leq 0.01$) for period 1 and highly significant ($P \leq 0.0001$) for periods 2 and 3. Observed color differences were larger in the first transfer period than in the second transfer period in all treatments

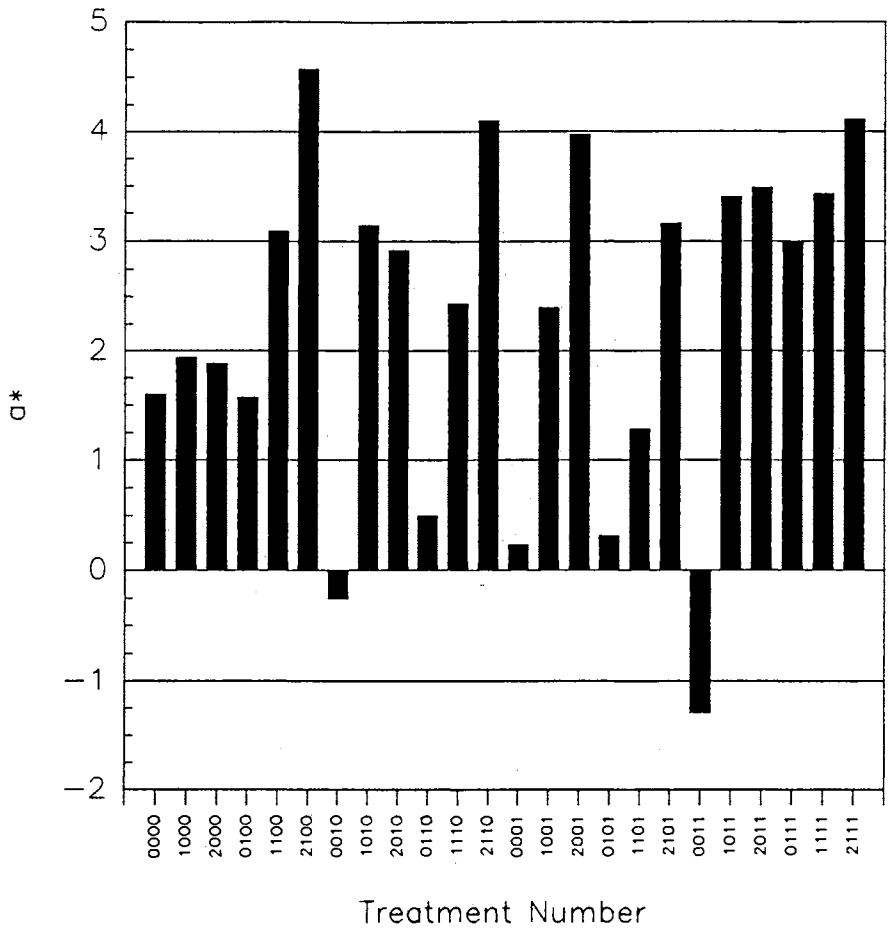

Figure 4. Differences of stimulus a* values at the final transfer period and stimulus a* values at the initial period of *Amaranthus hypochondriachus* calli as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 µM, 1 = 2.5 µM), and 2,4-D (0 = 10 µM, 1 = 15 µM).

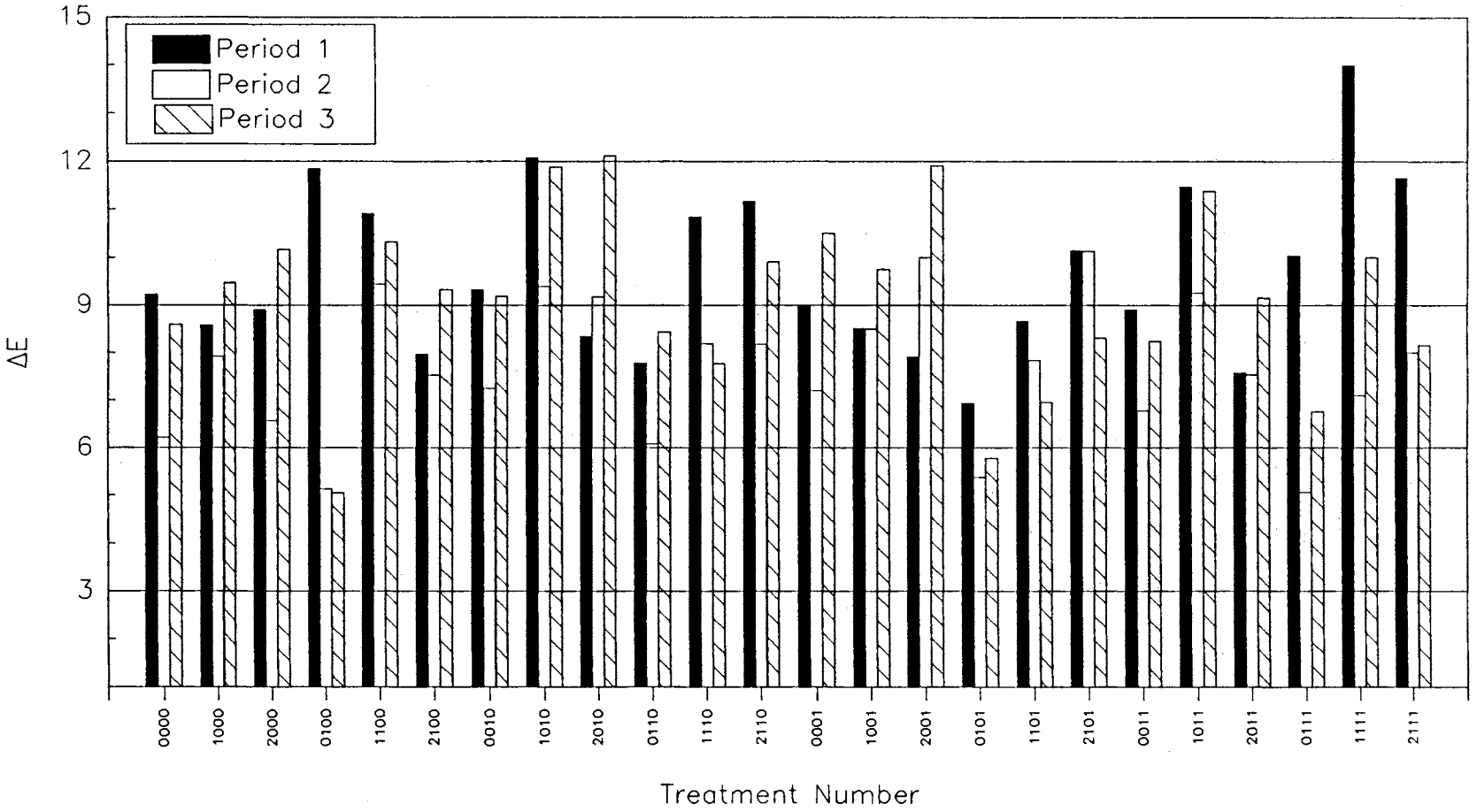

Figure 5. Total color difference (ΔE) of *Amaranthus hypochondriachus* calli as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

except 2010 and 2001 (Figure 5). These increasing ΔE values were due primarily to the increase in the stimulus L* values from the initial period to transfer period 1 (Table 5). The largest color difference occurred during period 1 for treatment 1111 (Table 8) and is attributed to significant ($P \leq 0.05$) increases in both the 'L' and 'b' values (Table 5 and Table 7, respectively) and a moderate increase in the 'a' values (Table 6) for that treatment. Treatments 0100, 2110, 0111, and 1111 exhibited significant ($P \leq 0.05$) decreases in observed color difference from period 1 to period 2 (Table 8).

Observed color differences were larger in the final transfer period (3) than in the second transfer period in all treatments except 0100, 1110, 1101, and 2101 (Figure 5). Only treatment 2000 exhibited a significant ($P \leq 0.05$) increase in color difference from period 2 to period 3 (Table 8). Observed color differences of treatments 0100, 1110, 0111, 1111, and 2111 are significantly ($P \leq 0.05$) decreased from period 1 to period 3 (Table 8). Observed color differences of treatments 2010 and 2001 were increased ($P \leq 0.05$) from period 1 to period 3 (Table 8). The significant increases in ΔE values for treatments 2010 and 2001 ($P \leq 0.05$) were due to larger tristimulus 'L' (Table 5), 'a' (Table 6), and 'b' (Table 7) values.

Hue Angle. Color, as more closely perceived by humans, is characterized by the hue angle (Shewfelt et al., 1988; Gnanasekharan et al., 1992; Trail et al., 1992). Figure 6

TABLE 8

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN TOTAL COLOR DIFFERENCE (ΔE) OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	9.22	6.21	8.59	3.08
0100	55.56	2.50	0.00	10.00	11.84	5.13	5.05	2.74
0010	55.56	1.25	2.50	10.00	9.32	7.25	9.18	2.94
0110	55.56	2.50	2.50	10.00	7.78	6.08	8.43	2.96
0001	55.56	1.25	0.00	15.00	8.99	7.20	10.50	3.34
0101	55.56	2.50	0.00	15.00	6.94	5.38	5.78	1.92
0011	55.56	1.25	2.50	15.00	8.90	6.78	8.23	3.37
0111	55.56	2.50	2.50	15.00	10.03	5.06	6.76	2.43
1000	111.11	1.25	0.00	10.00	8.57	7.92	9.47	2.58
1100	111.11	2.50	0.00	10.00	10.91	9.44	10.32	3.40
1010	111.11	1.25	2.50	10.00	12.07	9.39	11.88	2.89
1110	111.11	2.50	2.50	10.00	10.84	8.19	7.77	2.84
1001	111.11	1.25	0.00	15.00	8.51	8.49	9.75	3.23
1101	111.11	2.50	0.00	15.00	8.65	7.84	6.96	2.24
1011	111.11	1.25	2.50	15.00	11.46	9.25	11.38	3.44
1111	111.11	2.50	2.50	15.00	13.99	7.10	9.99	3.36
2000	166.67	1.25	0.00	10.00	8.90	6.57	10.16	2.64
2100	166.67	2.50	0.00	10.00	7.97	7.53	9.32	2.55
2010	166.67	1.25	2.50	10.00	8.34	9.17	12.11	3.62
2110	166.67	2.50	2.50	10.00	11.17	8.18	9.90	2.75
2001	166.67	1.25	0.00	15.00	7.91	9.99	11.90	2.95
2101	166.67	2.50	0.00	15.00	10.13	10.12	8.31	2.86
2011	166.67	1.25	2.50	15.00	7.57	7.53	9.15	2.56
2111	166.67	2.50	2.50	15.00	11.65	8.00	8.15	2.52
				LSD **	3.34	2.55	3.32	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

diagrams the Hunter 'ab' color space. An angle of 90° represents a yellow hue, an angle of 180° represents a green hue, an angle of 270° represents a blue hue, and an angle of 0° or 360° represents a red hue (Figure 6). Angles of importance in this study are those falling between 0° and 90°. Objects with angles near 90° are more yellow and become more orange-red to red as the angles decrease toward 0°. Objects with hue angles greater than 90° are becoming more green, and so on.

The results of the study exhibited a difference ($P \leq 0.0001$) in the mean hue angle between the treatments within each period. A summary of these results is presented in Table 9. The mean hue angle for the initial period was 82.69°. For the subsequent transfer periods, the mean hue angle decreased to 81.91° for period 1, 78.31° for period 2, and 77.92° for period 3; thus the hue angle became more red as the experiment continued. The treatment mean hue angles are plotted in Figure 7. This graph clearly shows a tendency toward more red calli by period 3 (Figure 7). Every treatment (except treatment 1101) exhibited a significant difference of at least $P \leq 0.05$ between periods (Table 9). There were no observed changes in the hue angle from period to period in treatment 1101 (Table 9). Treatments exhibiting the greatest changes in hue angle include 2100, 2110, 2001, 2101, and 2111 (Table 9). Each of these treatments contained a sucrose concentration of 166.67 mM (Table 1). Observed changes in the hue angles were

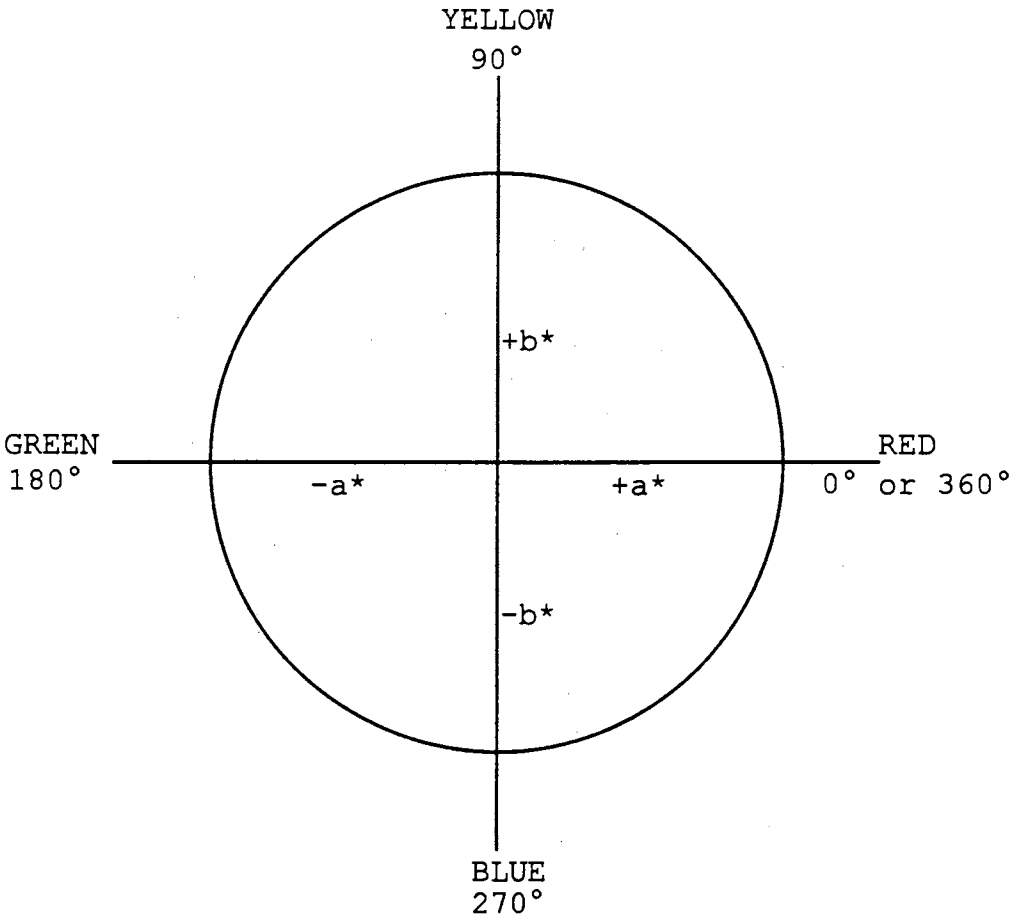

Fig. 6 - Diagram of the Hunter a,b color space. The hue angle indicates a change from red to yellow, yellow to green, green to blue, and from blue to red as it increases (counterclockwise).

TABLE 9

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN HUE ANGLE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 0	1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	80.13	86.92	82.35	77.45	2.77
0100	55.56	2.50	0.00	10.00	81.75	84.14	82.30	77.74	2.94
0010	55.56	1.25	2.50	10.00	80.43	85.85	76.92	82.88	2.76
0110	55.56	2.50	2.50	10.00	83.86	85.11	79.61	82.98	2.50
0001	55.56	1.25	0.00	15.00	82.23	86.12	80.06	83.81	2.40
0101	55.56	2.50	0.00	15.00	84.88	85.70	81.59	84.45	2.79
0011	55.56	1.25	2.50	15.00	79.50	82.99	75.86	84.29	3.24
0111	55.56	2.50	2.50	15.00	83.56	86.03	78.57	73.97	2.11
1000	111.11	1.25	0.00	10.00	80.47	82.73	80.13	77.55	2.98
1100	111.11	2.50	0.00	10.00	83.11	81.95	82.43	76.66	4.21
1010	111.11	1.25	2.50	10.00	83.41	77.97	77.36	77.61	3.05
1110	111.11	2.50	2.50	10.00	84.74	79.08	77.48	78.08	3.71
1001	111.11	1.25	0.00	15.00	82.67	82.88	79.23	77.65	2.47
1101	111.11	2.50	0.00	15.00	84.62	82.70	82.15	82.29	2.69
1011	111.11	1.25	2.50	15.00	82.28	80.32	74.91	76.66	2.34
1111	111.11	2.50	2.50	15.00	83.04	84.02	75.85	75.37	5.10
2000	166.67	1.25	0.00	10.00	81.95	78.58	76.44	79.17	2.47
2100	166.67	2.50	0.00	10.00	84.10	81.54	75.90	71.96	3.31
2010	166.67	1.25	2.50	10.00	81.78	76.58	75.81	76.65	2.69
2110	166.67	2.50	2.50	10.00	84.29	80.92	78.22	75.03	2.69
2001	166.67	1.25	0.00	15.00	82.09	78.03	76.81	73.55	2.54
2101	166.67	2.50	0.00	15.00	83.43	79.89	77.74	74.55	3.19
2011	166.67	1.25	2.50	15.00	83.81	77.74	77.26	75.47	2.37
2111	166.67	2.50	2.50	15.00	85.20	79.70	75.96	74.95	2.54
				LSD **	2.48	3.34	2.92	3.47	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

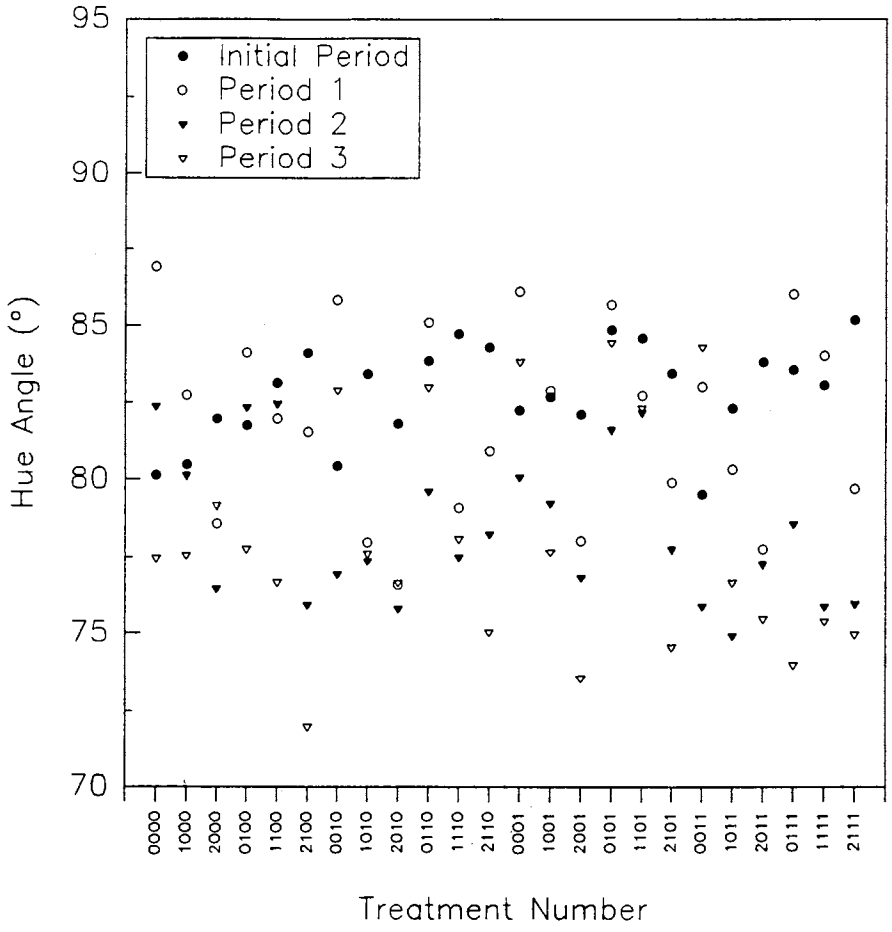

Figure 7. Mean hue angles of *Amaranthus hypochondriachus* calli as influenced by sucrose, phosphate, 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatment number is coded as follows: sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 µM, 1 = 2.5 µM), and 2,4-D (0 = 10 µM, 1 = 15 µM).

primarily due to more positive stimulus 'a' readings (Table 6).

A few treatments (0000, 1000, 0010, 0110, 0001, 0101, and 1101) did not demonstrate a significant ($P > 0.05$) difference from the initial period to the final transfer period in the hue angle (Table 9). Of these treatments, 0010, 0110, 0001, and 0101, also exhibited no significant ($P > 0.05$) increase in the stimulus 'a' values (Table 6). This suggests that an increase in the 'a' value can result in a more red hue angle. After all, a more positive 'a' value is more red in color, while a more negative 'a' value is more green in color (Figure 6).

The effect of each component level for each period is presented in Table 10. The concentration of sucrose (55.56 mM, 111.11 mM, and 166.67 mM) is highly effective ($P \leq 0.0001$) in periods 2 and 3, and somewhat effective ($P \leq 0.01$) in the initial and final period (Table 10). Calli grown on media with a 166.67 mM sucrose concentration had a more red hue angle ($P \leq 0.05$) than calli grown on media with either a 55.56 mM or 111.11 mM sucrose concentration (Table 10).

The influence of phosphate concentration (1.25 mM and 2.50 mM) on the hue angle was significant ($P \leq 0.01$) for period 2, and only significant at the $P \leq 0.05$ level for the initial period and periods 1 and 3 (Table 10). At transfer periods 1 and 2, calli grown on media containing 1.25 mM of phosphate possessed a more red hue angle ($P \leq 0.05$) while

TABLE 10

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) CONCENTRATION ON THE MEAN HUE ANGLE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Component	Concentration	Transfer Period				LSD *
		0	1	2	3	
Sugar	55.56	81.88	85.37	79.56	80.92	1.10
(mM)	111.11	82.95	81.40	78.61	77.69	1.21
	166.67	83.23	78.97	76.74	75.29	0.98
	LSD *	0.87	1.15	1.06	1.29	
Phosphate	1.25	81.39	81.73	77.76	78.56	0.89
(mM)	2.50	82.57	83.88	78.99	77.07	1.03
	LSD *	0.69	1.06	0.89	1.14	
BAP	0.00	82.51	82.59	79.71	77.98	0.95
(µM)	2.50	82.87	81.24	76.91	77.86	0.96
	LSD *	0.72	1.05	0.86	1.14	
2,4-D	10.00	82.38	81.74	78.68	77.80	0.97
(µM)	15.00	83.00	82.09	77.93	78.04	0.96
	LSD *	0.72	1.06	0.89	1.14	

* LSD - Least Significant Difference (0.05).

calli grown on media containing 2.50 mM of phosphate possessed a more red hue angle ($P \leq 0.05$) at transfer period 3 (Table 10).

The effect of BAP concentration (0.0 µM and 2.5 µM) on the hue angle was highly significant ($P \leq 0.0001$) at transfer periods 1 and 2 and not significant for the initial period and transfer period 3 (Table 10). In transfer period 2, a BAP concentration of 2.5 µM resulted in a more red hue angle ($P \leq 0.05$) (Table 10). The concentration of 2,4-D (10 µM and 15 µM) did not significantly affect the hue angle (Table 10).

A summary of ANOVA results for hue angle was developed for each component and their interactions (Table 11). The four-way interaction (SxPxBxD) was not significant ($P \leq 0.05$) (Table 11). The two-way interaction between sucrose and BAP (SxB) was significant ($P \leq 0.01$), and the addition of 2,4-D (SxBxD) resulted in a significant ($P \leq 0.01$) three-way interaction (Table 11). Phosphate interacted with BAP and 2,4-D (PxBxD) significantly ($P \leq 0.05$); however, its addition was not as effective as the addition of sucrose (Table 11).

Chroma. The chroma is the characteristic of an object's brightness (Gnanasekharan et al., 1992). The results of this study exhibited a difference($P \leq 0.001$) in the chroma between the treatments within each period. A summary of these results is presented in Table 12. The mean

TABLE 11

THE EFFECTS OF SUCROSE (S), PHOSPHATE (P), 6-BENZYLAMINOPURINE (B), AND 2,4-DICHLOROPHENOXYACETIC ACID (D) AND THEIR INTERACTIONS ON THE MEAN HUE ANGLE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Component	Statistical Significance
S	***
P	**
B	***
D	NS
SxP	NS
SxB	**
SxD	NS
PxB	NS
PxD	NS
BxD	**
SxPxB	NS
SxPxD	NS
SxBxD	**
PxBxD	*
SxPxBxD	NS

Significance of components and their interactions are indicated as: *** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$, NS - Not Significant.

chroma for the initial period was 14.95. For the subsequent transfer periods, the mean chroma increased to 20.87 for period 1, 19.72 for period 2, and 20.01 for period 3. Small values of chroma demonstrate weak, or grayish colors, while an increasing chroma demonstrates a tendency toward a relatively more vivid color. The mean chroma for each treatment at period 3 was significantly ($P \leq 0.05$) more vivid than the mean chroma at the initial period (Table 12). This coincides with the tendency toward a higher 'L' value of the treatments (Table 5).

Light Manipulation

Evaluation of Growth

Relative growth rates were evaluated and a summary of these results is presented in Table 13. At transfer period 1, calli which were grown in the dark and in green light treatments exhibited increases ($P \leq 0.05$) in relative growth rates compared with calli grown in white light and red light treatments (Figure 8). Calli treated with red light exhibited a lower ($P \leq 0.05$) relative growth rate (Figure 8). At transfer period 2, there was no significant difference in the relative growth rates of the treatments (Figure 8). By transfer period 3, calli treated with white light demonstrated the highest relative growth rate ($P \leq 0.05$), while calli grown in the dark maintained the lowest

TABLE 12

THE EFFECTS OF SUCROSE, PHOSPHATE, 6-BENZYLAMINOPURINE (BAP), AND 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE MEAN CHROMA (C*) OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment*	Sucrose (mM)	Phosphate (mM)	BAP (μM)	2,4-D (μM)	Transfer Period 0	1	2	3	LSD **
0000	55.56	1.25	0.00	10.00	13.92	16.35	16.88	18.25	1.70
0100	55.56	2.50	0.00	10.00	14.85	20.73	17.84	17.71	1.50
0010	55.56	1.25	2.50	10.00	14.83	19.35	18.72	17.12	1.78
0110	55.56	2.50	2.50	10.00	15.92	20.40	18.72	19.14	1.66
0001	55.56	1.25	0.00	15.00	14.68	18.35	17.92	19.78	1.81
0101	55.56	2.50	0.00	15.00	16.62	20.25	19.42	19.37	1.50
0011	55.56	1.25	2.50	15.00	13.93	18.23	17.34	15.79	1.77
0111	55.56	2.50	2.50	15.00	16.96	22.45	19.09	17.75	1.46
1000	111.11	1.25	0.00	10.00	15.03	20.14	20.20	20.67	1.45
1100	111.11	2.50	0.00	10.00	14.10	22.80	21.15	21.54	2.21
1010	111.11	1.25	2.50	10.00	14.92	23.86	20.90	23.10	1.98
1110	111.11	2.50	2.50	10.00	15.89	24.11	20.81	19.01	1.92
1001	111.11	1.25	0.00	15.00	14.10	19.24	19.73	20.16	2.17
1101	111.11	2.50	0.00	15.00	16.33	23.22	22.57	20.26	1.67
1011	111.11	1.25	2.50	15.00	13.09	22.27	20.34	22.30	2.12
1111	111.11	2.50	2.50	15.00	16.39	26.32	19.99	22.20	1.72
2000	166.67	1.25	0.00	10.00	14.28	19.45	18.82	20.54	1.63
2100	166.67	2.50	0.00	10.00	15.31	21.01	20.71	20.38	1.54
2010	166.67	1.25	2.50	10.00	14.12	19.16	20.36	21.43	1.93
2110	166.67	2.50	2.50	10.00	15.96	24.40	22.12	22.28	1.70
2001	166.67	1.25	0.00	15.00	13.69	17.60	19.72	20.65	1.52
2101	166.67	2.50	0.00	15.00	15.55	22.26	22.13	19.17	1.73
2011	166.67	1.25	2.50	15.00	14.41	18.26	19.10	19.93	1.57
2111	166.67	2.50	2.50	15.00	16.26	25.29	20.52	21.40	1.96
				LSD **	1.57	2.15	1.64	2.04	

* Treatment is coded according to the amount of the component as follows: Sucrose (0 = 55.56 mM, 1 = 111.11 mM, 2 = 166.67 mM), Phosphate (0 = 1.25 mM, 1 = 2.50 mM), BAP (0 = 0.0 μM, 1 = 2.5 μM), and 2,4-D (0 = 10 μM, 1 = 15 μM).

** LSD - Least Significant Difference (0.05).

TABLE 13

THE EFFECT OF LIGHT SOURCE ON THE RELATIVE GROWTH RATES OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period				LSD *
	1	2	3	4	
White Light	0.036	0.104	0.104	0.046	0.017
Dark	0.060	0.107	0.055	0.067	0.013
Green Light	0.060	0.097	0.076	0.056	0.014
Red Light	0.007	0.093	0.076	0.055	0.016
LSD *	0.017	0.015	0.017	0.008	

* LSD - Least Significant Difference (0.05).

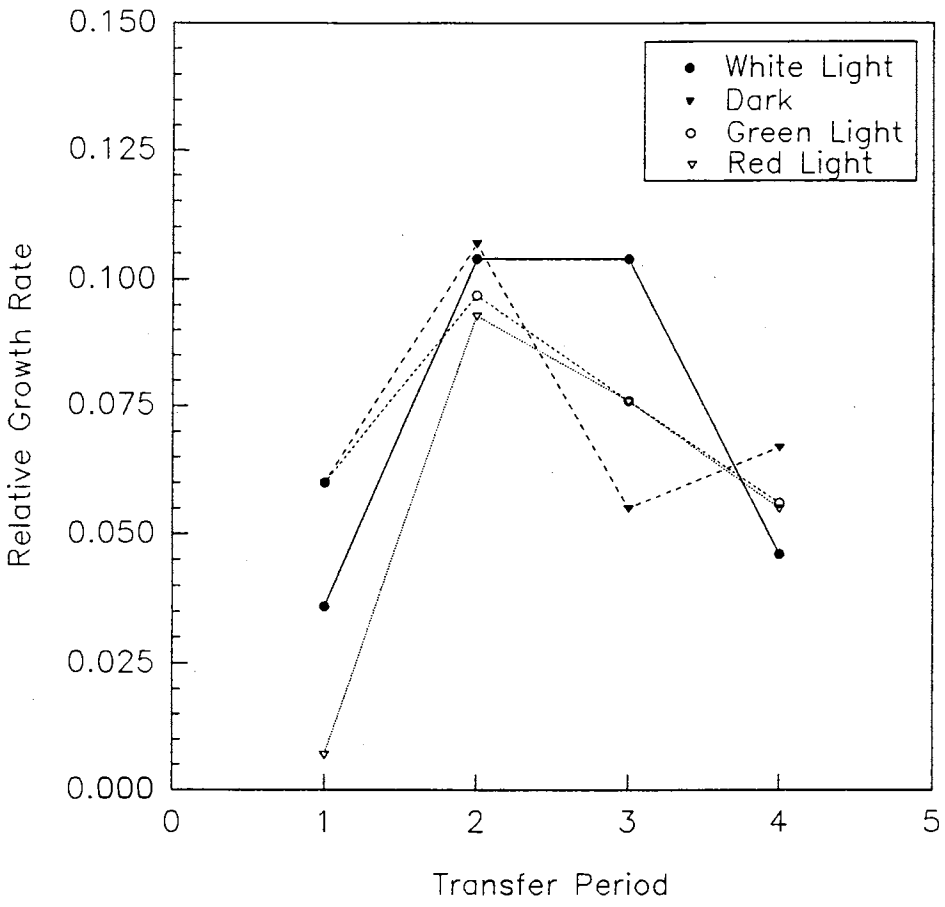

Figure 8. Relative growth rates of *Amaranthus hypochondriachus* calli for each transfer period as influenced by light source (white light, no light, green monochromatic light, and red monochromatic light).

($P \leq 0.05$) relative growth rate (Figure 8). At transfer period 4, calli grown in the dark exhibited the highest ($P \leq 0.05$) relative growth rates, while calli grown in white light exhibited the lowest ($P \leq 0.05$) relative growth rate (Figure 8).

The relative growth rate of calli grown in white light increased from period 1 to period 2 ($P \leq 0.05$) and then decreased ($P \leq 0.05$) by period 4 (Table 13). The relative growth rate of calli grown without any light also increased ($P \leq 0.05$) from period 1 to period 2, yet decreased to the level of period 1 and maintained this level through period 4 (Table 13). The relative growth rate of calli grown in green light was the highest at period 2, decreased significantly ($P \leq 0.05$) by period 3, and decreased significantly ($P \leq 0.05$) once again by period 4 (to the same level of period 1). Calli grown in the red light exhibited the highest relative growth rate at period 2, with steady decreases ($P \leq 0.05$) through each successive period (Table 13).

Evaluation of Color

Values of tristimulus L*, a*, and b*, were measured and summaries of these results are presented in Table 14, Table 15, and Table 16, respectively. Initially, the light sources highly affected the 'L' values ($P \leq 0.0001$), however, at transfer periods 1 and 2, the changes in the 'L'

values were slightly significant ($P \leq 0.01$). The change in the 'L' value for period 3 was significant ($P \leq 0.05$), and increased in significance by period 4 ($P \leq 0.01$). Each treatment exhibited a significant difference in the 'L' value between periods of at least $P \leq 0.01$ (Table 14). Calli grown in the presence of red light exhibited a significant difference in the 'L' value of $P \leq 0.001$ (Table 14). In each treatment, the final transfer period is significantly ($P \leq 0.05$) lighter than in the initial period (Table 14).

The differences in the stimulus a* values were initially highly significant ($P \leq 0.0001$), however, the 'a' values were only significant ($P \leq 0.01$) for periods 1 and 2. Transfer period 3 and 4 showed no significant differences in the 'a' values. Calli grown in the presence of red light and in the absence of light exhibited increases ($P \leq 0.05$) in the 'a' values from the initial period to the final period (Table 15). Stimulus a* values of calli grown in white light decreased ($P \leq 0.05$) from the initial period to period 1 and maintained at that level for the remainder of the experiment (Table 15). Calli grown in white light exhibited a decrease ($P \leq 0.05$) in the 'a' value from the initial period to transfer periods 1 and 2 (Table 15). Transfer periods 3 and 4 were not significantly different ($P > 0.05$) from the successive periods (Table 15).

The difference in the stimulus b* values were initially only significant at the $P \leq 0.05$ level, yet increased in

TABLE 14

THE EFFECT OF LIGHT SOURCE ON THE MEAN L* VALUE
OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period					LSD *
	0	1	2	3	4	
White Light	33.41	39.58	36.44	39.36	39.90	3.40
Dark	39.73	43.99	43.71	42.51	47.06	3.39
Green Light	37.14	41.71	41.39	42.06	44.42	3.11
Red Light	33.44	39.19	41.72	43.12	45.23	3.14
LSD *	2.37	2.70	3.75	3.11	3.76	

* LSD - Least Significant Difference (0.05).

TABLE 15

THE EFFECT OF LIGHT SOURCE ON THE MEAN a* VALUE
OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period					LSD *
	0	1	2	3	4	
White Light	5.73	3.42	3.92	4.12	4.59	1.67
Dark	2.50	2.21	2.09	3.69	4.27	1.46
Green Light	3.98	2.17	3.68	4.20	4.71	1.10
Red Light	4.34	3.74	2.60	3.83	5.65	1.11
LSD *	0.94	1.06	1.33	1.70	1.42	

* LSD - Least Significant Difference (0.05).

TABLE 16

THE EFFECT OF LIGHT SOURCE ON THE MEAN b* VALUE
OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period					LSD *
	0	1	2	3	4	
White Light	19.08	20.91	20.84	22.82	21.14	2.00
Dark	19.49	19.84	18.00	18.16	20.67	2.37
Green Light	19.53	24.12	27.31	26.46	27.81	2.40
Red Light	17.54	20.18	24.36	27.23	26.71	2.16
LSD *	1.52	2.09	2.45	2.72	2.29	

* LSD - Least Significant Difference (0.05).

significance ($P \leq 0.01$) by transfer period 1. Transfer periods 2, 3, and 4, each exhibited highly significant ($P \leq 0.0001$) differences in 'b' values. Calli grown in the absence of light did not exhibit a significant ($P \leq 0.05$) difference between periods, while calli treated with white light exhibited some differences ($P \leq 0.01$) (Table 16). Calli treated with red and green light exhibited highly significant differences in 'b' values between certain periods ($P \leq 0.0001$) (Table 16). Calli treated with white light, green light, and red light exhibited significantly higher ($P \leq 0.05$) 'b' values at transfer period 4 than 'b' values at the initial period (Table 16).

Total Color Difference. ΔE or the total color difference observed from the initial period was determined for each subsequent transfer period (Figure 9). The difference between treatments was not significant for period 1, significant ($P \leq 0.05$) for period 2, highly significant ($P \leq 0.0001$) for period 3, and slightly significant ($P \leq 0.01$) for period 4 (Figure 9). Observed color differences were larger in period 3 for calli treated with white light, a significant ($P \leq 0.05$) increase over period 2 (Table 17). The remaining treatments exhibited a significant ($P \leq 0.05$) increase in observed color differences from period 1 to period 4 (Table 17). In periods 2, 3, and 4, calli treated with red light exhibited the largest mean color differences, and calli treated with green light exhibited the second

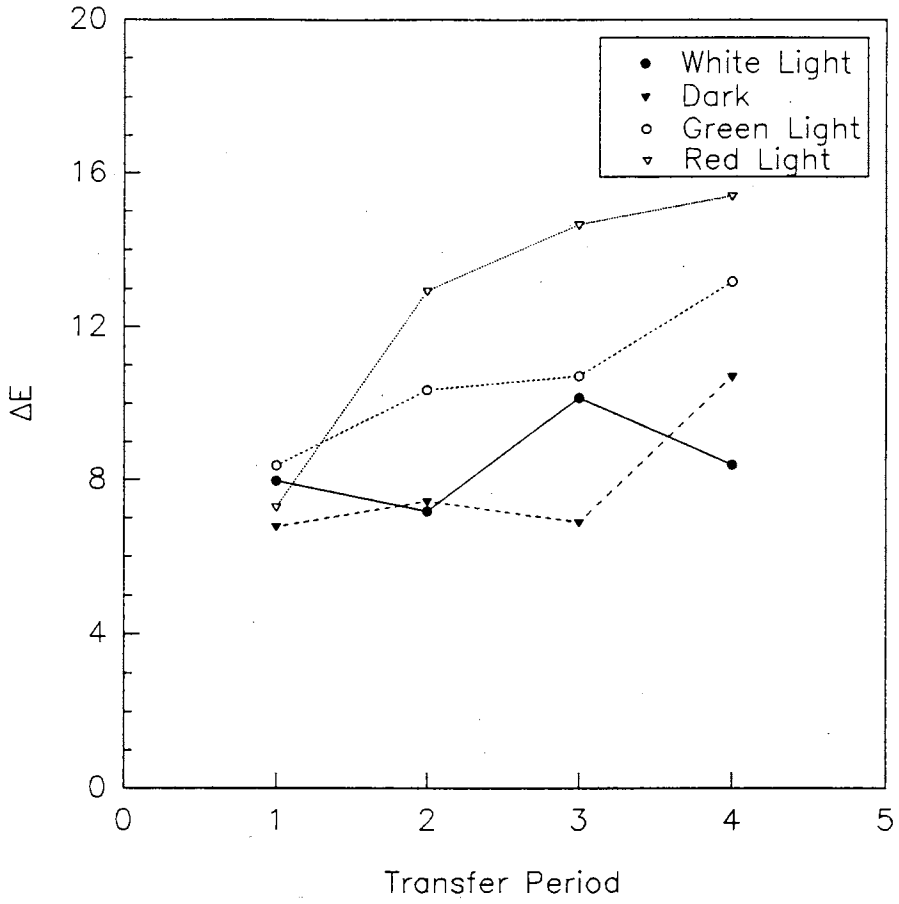

Figure 9. Total color difference (ΔE) of *Amaranthus hypochondriachus* calli for each transfer period as influenced by light source (white light, no light, green monochromatic light, and red monochromatic light).

TABLE 17

THE EFFECT OF LIGHT SOURCE ON THE MEAN TOTAL COLOR DIFFERENCE (ΔE) OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period				LSD *
	1	2	3	4	
White Light	7.97	7.17	10.15	8.39	2.96
Dark	6.76	7.44	6.89	10.70	2.58
Green Light	8.37	10.35	10.71	13.18	3.03
Red Light	7.29	12.94	14.65	15.40	4.15
LSD *	2.20	3.40	3.22	3.61	

* LSD - Least Significant Difference (0.05).

largest color differences for the same periods (Figure 9). Observed color differences of calli treated with red and green light were due to larger 'a' and 'b' values than those exhibited by calli treated with white light and no light (Tables 15 and 16, respectively).

Hue Angle. The results of the study exhibited a difference ($P \leq 0.0001$) in the mean hue angle between the treatments within the initial period and transfer period 1. There was no significant difference in the mean hue angle for transfer periods 2, 3, and 4. The mean hue angle for the initial period was 77.59°, relatively more red than the mean hue angle for transfer period 1 (82.28°). The mean hue angle remained at the level of transfer period 1 through transfer period 2 (82.36°). By transfer period 3, the mean hue angle became relatively more red (80.84°), and increasingly more red by transfer period 4 (78.97°).

Calli treated with red and green light exhibited highly significant differences ($P \leq 0.0001$) in the mean hue angle (Table 18). Calli treated with white light and no light exhibited differences ($P \leq 0.05$) in the mean hue angle (Table 18). The hue angle of calli treated with white light increased significantly ($P \leq 0.05$) from the initial period to transfer period 1, and maintained the significance level through the entire experiment (Table 18). The hue angle of calli treated with no light exhibited no significant difference for the initial period and transfer periods 1, 2,

TABLE 18

THE EFFECT OF LIGHT SOURCE ON THE MEAN HUE ANGLE OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period					LSD *
	0	1	2	3	4	
White Light	73.22	80.53	79.47	80.03	78.06	4.49
Dark	82.86	84.28	84.04	80.50	79.26	3.87
Green Light	78.30	84.91	82.34	80.77	80.27	2.62
Red Light	75.99	79.40	83.59	82.04	78.28	2.80
LSD *	2.95	2.82	3.55	4.55	2.98	

* LSD - Least Significant Difference (0.05).

and 3; however, transfer period 4 was significantly ($P \leq 0.05$) more red than transfer periods 1 and 2 (Table 18). Calli treated with green light exhibited a significant ($P \leq 0.05$) increase in the hue angle from the initial period to transfer period 1, and became significantly more red by transfer periods 3 and 4 (Table 18). The hue angle of calli treated with red light steadily ($P \leq 0.05$) increased from the initial period to transfer period 2, remained at that significance level for transfer period 3, and then significantly ($P \leq 0.05$) decreased (becoming relatively more red) at transfer period 4 (Table 18).

Chroma. The results of this study exhibited a difference ($P \leq 0.001$) in the mean chroma between the treatments for transfer periods 2, 3, and 4. The initial period demonstrated no significant differences in the mean chroma between treatments, however, transfer period 1 demonstrated a significant difference ($P \leq 0.01$) in the mean chroma between treatments. A summary of these results is presented in Table 19.

The mean chroma difference was not significant between periods for calli treated without light, slightly significant ($P \leq 0.05$) for calli treated with white light, and highly significant ($P \leq 0.0001$) for calli treated with green and red light (Table 19). In both green and red treatments, the calli demonstrated a relatively more vivid chroma at transfer period 4 than the chroma at the initial

TABLE 19

THE EFFECT OF LIGHT SOURCE ON THE MEAN CHROMA (C*)
OF AMARANTHUS HYPOCHONDRIACHUS CALLI

Treatment	Transfer Period					LSD *
	0	1	2	3	4	
White Light	19.97	21.30	21.41	23.51	21.74	1.98
Dark	19.68	20.02	18.18	18.73	21.23	2.51
Green Light	19.99	24.28	27.64	26.86	28.23	2.38
Red Light	18.16	20.56	24.57	27.53	27.35	2.17
LSD *	1.50	2.10	2.43	2.73	2.42	

* LSD - Least Significant Difference (0.05).

period (Table 19). The increased vividness of the mean chroma for these treatments coincides with the tendency toward higher 'L' values and similarly higher 'b' values of the treatments (Tables 14 and 16, respectively). Calli treated with white light exhibited a significantly ($P \leq 0.05$) more vivid chroma at transfer period 3 than the preceding periods (Table 19).

CHAPTER IV

DISCUSSION

In this study, an efficient method of plant tissue culture to produce betalain from *Amaranthus hypochondriachus* was identified. An efficient method of producing the betalain pigment through plant tissue culture can lead to a cost effective use of this "natural" color in the food industry. Seeds of *Amaranthus hypochondriachus* were evaluated for callus initiation on MS media and the effect of 2,4-D was determined. Media with various levels of sucrose, phosphate, BAP, and 2,4-D, were also evaluated for growth and color production of the calli. The effect of light wavelength on growth and color production of the *Amaranthus hypochondriachus* calli was also determined.

The level of 2,4-D (5.7 µM and 22.6 µM) did not affect initiation (90.37% and 88.03%, respectively) of the *Amaranthus hypochondriachus* seeds; however, callus condition was greatly affected. Calli initiated on media with a 2,4-D concentration of 5.7 µM exhibited an abundance of root growth. The root growth was absent in calli initiated on media with a 2,4-D concentration of 22.6 µM. It is believed that the ideal 2,4-D concentration lies somewhere in between the two experimental levels, and future experimentation

could identify this concentration. Relative growth rates of calli grown on media with a 2,4-D concentration of 5.7 µM were larger, yet the rate can be attributed to increased root growth. Relative growth rates of calli grown on media with a concentration of 22.6 µM was slightly lower, yet increased when transferred to media with a 2,4-D concentration of 10 µM. Calli grown on media with a 2,4-D concentration of 5.7 µM were hairy with roots and not clear, while calli grown on media with a 2,4-D concentration of 22.6 µM were compact, clear, and exhibited no root or shoot growth. Davis and Guenzi (1988) developed a tissue culture methodology for callus induction and plant regeneration of *Amaranthus hypochondriachus*. They found shoot formation with MS media and a 2,4-D concentration of 5.7 µM, and maximized shoot formation by lowering the 2,4-D concentration to 0.45 µM. They also noted patches of red betalain and green chlorophyll pigments in the initiated calli. Due to its inclination to show pigmentation (Davis and Guenzi, 1988), *Amaranthus hypochondriachus* was chosen for this research.

Relative growth rates were determined for both experiments (media manipulation and light manipulation). The level of sucrose was very important in the rate of growth of calli. Sucrose is used in the medium as a source of reduced carbon for the growing calli; however, too much sucrose can reduce the water potential of the media, resulting in a reduced relative growth rate of the calli.

This is indicated by the observations that calli grown on medium with 111.11 mM sucrose content exhibited a much higher mean relative growth rate while calli grown on medium containing 166.67 mM sucrose content exhibited a much lower mean relative growth rate. A combination of sucrose and BAP were very important in increasing the relative growth rate. Calli grown on media with a BAP concentration of 2.5 μM exhibited higher mean relative growth rates. A combination of 111.11 mM sucrose and 2.5 μM BAP resulted in higher mean relative growth rates. Once again, medium containing 111.11 mM sucrose proved to be a sufficient amount and not toxic to the growing calli (as in medium containing 166.67 mM sucrose). It has been shown (Rau and Forkmann, 1986) that increased levels of phosphate in the medium resulted in higher growth rates. The level of phosphate was moderately important in increasing the growth rate of calli. The level of 2,4-D seemed to be most effective in combination with sucrose and BAP. The two treatments exhibiting the highest relative growth rates (1110 and 1111) both contain 111.11 mM sucrose, 2.5 mM phosphate, and 2.5 μM BAP. This combination of nutrients supplies the needed carbon and phosphate for growth of calli.

The effect of light wavelength on relative growth rates was not easily determined. Prior research has been done mostly with seedlings, not calli. The mean relative growth rates for white light, dark, and green light treatments were equal (0.073), with the mean relative growth rate for red

light being considerably lower (0.058) for the experiment. However, relative growth rates were significantly higher for period 2 compared to period 1. This is due to a possible adjustment of the calli to their new environment in the dark, green light, and red light treatments; however, this does not explain the significant increase in the relative growth rate in the white light treatment from period 1 to period 2.

Color was evaluated, non-destructively, with a colorimeter. The tristimulus L*, a*, and b*, values were used to compute the hue angle, total color difference, and chroma. Plant pigments, such as betalain, are secondary metabolites and are produced in higher quantities when the plant is stressed (Pierik, 1987). Insufficient nutrient requirements would be a cause of stress in the calli. This would result in reduced relative growth rates and increased color production.

Davis and Guenzi (1988) noticed red pigmentation in *Amaranthus hypochondriachus* calli plated on media with 2.5 µM BAP. The highest relative growth rates occurred in calli on media with 111.11 mM sucrose and 2.5 µM BAP. These calli are apparently not stressed. However, the lowest relative growth rates occurred in calli on media with 166.67 mM sucrose and 2.5 µM BAP. Calli growth on this media was probably inhibited due to the lower water potential of the media. These calli exhibit signs of stress; a failure to maintain a high relative growth rate and exhibiting an

increased production of the secondary metabolite, betalain. Calli grown on media containing 166.67 mM sucrose consistently had more positive 'a' values, indicating a more red color. These large 'a' values contributed to a more red hue angle, also. This is in agreement with Kang et al. (1992) who reported betalain production in red beets was greatest with 6% sucrose and 200 mg/L phosphate during suspension cell culture. Once again, the concentration of 2,4-D (10 µM and 15 µM) had no apparent effect on the production of betalain.

It has been shown that betalain production in *Amaranthus sp.* seedlings is stimulated by light (French et al., 1973), and is under phytochrome control (Köhler, 1972; French et al., 1973). Kang et al. (1992) reported increased betalain production with monochromatic green light as compared to red or blue monochromatic light. Red pigment patches appeared on calli treated with green monochromatic light earlier in the experiment than any callus of other treatments. However, the red patches were not maintained.

Calli treated with red light exhibited the largest total color difference (ΔE) by the final transfer period, and as would be expected, the calli treated with no light maintained the smallest mean ΔE. This study suggests that red monochromatic light be used to increase production of betalain. This coincides with the lower relative growth rates of calli treated with red light. These calli might

possibly be stressed, thus exhibiting a lower relative growth rate and an apparent increase in color production.

CHAPTER V

CONCLUSIONS

The color of food is very important. Research in the production of natural pigments for use as food colorants is on the leading edge of technology and is a requirement for the future. Plant tissue culture can be used efficiently in this research.

This study showed that *Amaranthus hypochondriachus* is an excellent raw material for plant tissue culture. Callus initiation occurred at a high percentage (approximately 90%) and responded well to media nutrients. The concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) should be maintained near 10 µM to 15 µM to prevent excessive root growth (as occurred with 5.7 µM 2,4-D) and to prevent degenerated growth (as occurred with 22.6 µM 2,4-D). MS media proved to be an effective media for proper nutrient requirements of initiated calli.

Nutrient components (sucrose, phosphate, 6-benzylaminopurine (BAP)), and 2,4-D proved to be important for growth and color production. For best growth, the medium should contain 111.11 mM sucrose, 2.50 mM phosphate, and 2.5 µM BAP. The concentration of 2,4-D proved to be more important in retarding extraneous root growth;

therefore, concentrations of 10 µM to 15 µM 2,4-D should be maintained. Requirements for maximum color production would include 166.67 mM sucrose, 2.50 mM phosphate, and 2.5 µM BAP. These nutrient levels apparently stress the calli (by reducing the water potential of the media), thereby, decreasing the relative growth rate and increasing the color production. The concentration of 2,4-D did not affect color production. Once again, though, the presence of 2,4-D at concentrations of 10 µM to 15 µM should be maintained to prevent excessive root growth.

Wavelength of light (white light, no light, green monochromatic light, and red monochromatic light) also proved to be important for growth and color production. All treatments, except red monochromatic light, exhibited similar relative growth rates. The relative growth rate of calli treated with red light were lower. These calli expressed symptoms of stress, leading to increased production of betalain. Calli treated with white light also maintained color production.

The results of this research indicate that the optimum conditions for color production were: 166.67 mM sucrose, 2.50 mM phosphate, 2.5 µM BAP, 10 µM to 15 µM 2,4-D (to prevent excessive root growth), and red monochromatic light. The color production (as with most secondary metabolites) of calli is increased with stress. The above growing conditions provide the needed stress for color production.

Further research is needed to further optimize tissue culture conditions to increase growth rate and betalain biosynthesis. Further study is also needed to convert this process from a static culturing to a more efficient continuous process, as in a bioreactor. In a continuous process, harvesting of the color will not interfere with growth and production of betalain. A continuous process is also more economical.

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APPENDIX

COMPOSITION OF MS MEDIA

MAJOR SALTS

Substance	mM
KNO_3	18.79
KH_4NO_3	15.71
$CaCl_2*2H_20$	3.96
$MgSO_4*7H_2O$	3.07
KH_2PO_4	1.25
$FeSO_4*7H_2O$	0.18
Na_2*EDTA	0.11

MINOR SALTS

Substance	µM
$MnSO_4*H_20$	112.58
H_3BO_3	98.30
$ZnSO_4*7H_2O$	65.04
KI	4.82
$Na_2MoO_4*2H_2O$	1.02
$CoCl_2*6H_20$	0.19
$CuSO_4*2H_2O$	0.16

VITA

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