EFFECTS OF LIGHT, CARBON DIOXIDE, AND HORMONE LEVELS ON TRANSFORMATION TO PHOTOAUTOTROPHY OF SUGARCANE SHOOTS IN MICROPROPAGATION

H. Erturk, P. N. Walker

ABSTRACT. *Sugarcane (Saccharum spp.) shoots were transferred from a heterotrophic micropropagation environment and cultured on sugar-free Murashige and Skoog (MS) salts medium in the controlled atmosphere of a growth chamber. The purpose was to achieve photoautotrophic shoot culture to be used for micropropagation. Effects on the shoots were tested for three factors: carbon dioxide concentration, light level, and the hormone concentration of the growth medium. Factorial design was applied for the experiment such that all combinations of high and low factor levels were utilized, and the medium level of each factor level constituted the middle point of the design. All shoots were observed to become yellowish in color and lose vigor in the sugarless environment, although the successful treatments regained their color and vigor. Average dry weights of shoots per vessel were recorded at the end of two weeks as a quantitative measure of transformation of shoots to photoautotrophy. Light and CO_2 levels were found to have statistically significant and positive effects. The negative effect of hormone concentration was insignificant.*
Keywords. *Micropropagation, Tissue culture, Photoautotrophic, Controlled environment.*

In conventional micropropagation, cultures are heterotrophic or mixotrophic and utilize sugar in the medium as all or part of their energy source. Aseptic culture conditions are required because the presence of sugar in the medium will result in rapid growth of microorganisms in the case of such contamination. Removal of sugar from the medium, i.e., growing shoots photoautotrophically, can perhaps allow micropropagation under septic conditions. Micropropagation under septic conditions, compared to aseptic conditions, might lead to reduced cost per plantlet produced, even if the micropropagule production rates are lower for septic conditions. Recently, the necessity of sugar in conventional micropropagation media and possibilities of eliminating sugar from the media have been analyzed (Kurata and Kozai, 1992; Hayashi et al., 1992). It has been suggested that, in general, plantlets will develop photoautotrophy and grow faster under higher CO_2 and higher photosynthetic photon flux (PPF) conditions (Kozai and Iwanami, 1988; Kozai et al., 1988).

Based on previous photoautotrophic growth studies, the main hypothesis for this research was that photoautotrophic micropropagation of sugarcane shoots can be achieved by providing a suitable environment, e.g., high light and high CO_2 conditions. The main objective of the study in whole was to demonstrate photoautotrophic micropropagation of sugarcane under septic conditions. The effects of environmental conditions on the shoots during transformation to photoautotrophy are presented in this article. After successful demonstration of this transformation, later studies can focus on improving the micropropagation multiplication and growth under photoautotrophic and septic conditions.

The effects of CO_2 enrichment and high light on the growth of shoots in photoautotrophic and mixotrophic environments have been studied by several researchers: Kozai and Iwanami (1988) with carnation; Kozai et al. (1988) with potato; Kozai et al. (1990) with *Cymbidium*; Fujiwara et al. (1987) with foliage; Cournac et al. (1991) with potato, Kozai (1991b) with strawberry. They found that CO_2 enrichment and high light stimulated the growth of plants cultured photoautotrophically.

Walker et al. (1991) studied the optimal environmental conditions for heterotrophic sugarcane shoots in Stage 2 (multiplication stage), based on the cost of production. The optimal sucrose content in the medium was found to be 2% at a temperature of 31°C, with six initial shoots per vessel, a photosynthetic photon flux of 200 $\mu mol\ m^{-2}\ s^{-1}$, and three medium changes over the 14-day growth cycle. They also tested whether CO_2 enrichment would be beneficial for plants to grow photoautotrophically in the absence of sugar (sucrose) normally used in micropropagation media. They found that ventilation with CO_2 enriched air (1400 $\mu mol\ mol^{-1}$) did not improve plant growth with or without sucrose in the medium.

Kozai (1991b) suggested that the use of plant growth regulating substances, vitamins, and other organic compounds could be minimized by growing plants photoautotrophically because some of these will be produced endogenously by the plants.

The objective of the study reported here was to test the effects of environmental conditions, i.e., CO_2 concentration, light level and hormone concentration of the medium, on the transformation to photoautotrophy in sugar-free medium of

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sugarcane shoots previously micropropagated in medium containing sugar.

MATERIALS AND METHODS

GROWTH MEDIUM

Solid MS (Murashige and Skoog, 1962) salts medium was used. A stock vitamin solution was prepared with concentrations of thiamine 0.1 mg l^{-1}, pyridoxine 0.5 mg l^{-1}, nicotinic acid 0.5 mg l^{-1}, glycine 0.2 mg l^{-1}, and myo-inositol 100 mg l^{-1}. The hormones kinetin and benzylaminopurine (BAP) were prepared as stock solutions each with a concentration of 0.1 mg mL^{-1}.

The sugar-medium was prepared as follows: sucrose 20 g l^{-1} and MS Salts 4.3 g l^{-1} were dissolved in distilled water. Two milliliter vitamin, 1 mL kinetin, and 2 mL BAP stock solutions were put in 1 l of medium. Finally, 1.1 to 1.2 g gelling agent, Phytagel™, was added. The sugarless medium was prepared in the same way except no sucrose was added. The medium was autoclaved for 20 min at 121°C. The medium was then cooled to about 50°C and dispensed aseptically into previously sterilized 400 mL Magenta vessels (Magenta Corp., Chicago, Ill.). Each vessel received 90 to 100 mL of warm medium that cooled and gelled in the vessels.

STOCK SUBCULTURING PROCEDURE

The stock culture of sugarcane (*Saccharum* spp.) cultivar 85-845 used for this research was a gift of Crop Genetics (Baton Rouge, La.). Sugarcane shoots were multiplied (Stage 2 of micropropagation), according to the procedures applied by Crop Genetics. Sugar-medium was used for stock culture and the cultures were therefore heterotrophic. The subculturing procedure took place by simply taking the shoot clumps out of their vessels, trimming the tops, cutting the bigger clumps gently into smaller clumps with a scalpel and transferring these into vessels with fresh medium. Two to four clumps were placed in each vessel. The shoots were typically moved to fresh medium within two weeks and subcultured within a month. In moving the shoots to fresh medium, all the shoots from each vessel were moved to a vessel containing fresh medium, working under an aseptic hood and disturbing the shoot clumps as little as possible.

CULTURE CONDITION

The sugarcane shoots used in the treatment vessels came from the heterotrophic stock culture and were all subcultured about one month before the experiment and again on the day the experiment began. On the day the experiment began, the subcultured shoots were transferred to vessels with four shoot clumps per vessel. A typical clump size was four or five shoots. During the experiment the shoot clumps were moved to fresh medium weekly using the procedure given above.

GROWTH CHAMBER

The light level in the growth chamber (Tenney Inc. Model No: TH650200, a reach-in chamber) was obtained using 40 W cool white fluorescent lamps. The chamber had a single shelf, with 115 × 115 cm shelf area, where vessels were placed. The light level, measured as PPF at the center point of the shelf using a Li-Cor LI-185A quantum sensor, was ~150 µmol m^{-2} s^{-1}. The PPF varied due to location on the shelf. The temperature in the growth chamber was controlled and maintained at 31 ±2°C. Shading and placement of vessels in the growth chamber controlled the light level in each vessel. The shading was obtained by covering the vessels with pieces of shade cloth cut to the size of the lids and taped to the top of the vessels.

CO_2 ENRICHMENT OF THE AIR

Air from a compressor was channeled and mixed with pure CO_2 to obtain three streams of air with CO_2 concentrations of 2200, 1275, and about 350 µmol mol^{-1}. The latter was ambient air with no CO_2 added. Enrichment was achieved by mixing air with pure CO_2 from a cylinder metered by a single channel peristaltic pump with a speed controller (fig. 1). The CO_2 enriched air was pumped through water-filled gas-washing bottles for humidification.

The humidified air was pumped to the vessels at the rate of 0.1 l min^{-1} using multihead peristaltic pumps. The air streams then entered the Nalgene™ bottles (500 mL volume) for trapping liquid water which might come from the gas washing bottles. Filters (hydrophobic, glass fiber filter 0.2 µm pore size) inside the Nalgene bottles were used to eliminate the air-borne bacteria and other microparticles. Magenta vessels were modified to receive air through flexible tubing (fig. 2). The air exited around the vessel lid. Gas chromatography was used to verify that desired CO_2 concentrations entering the vessels were achieved.

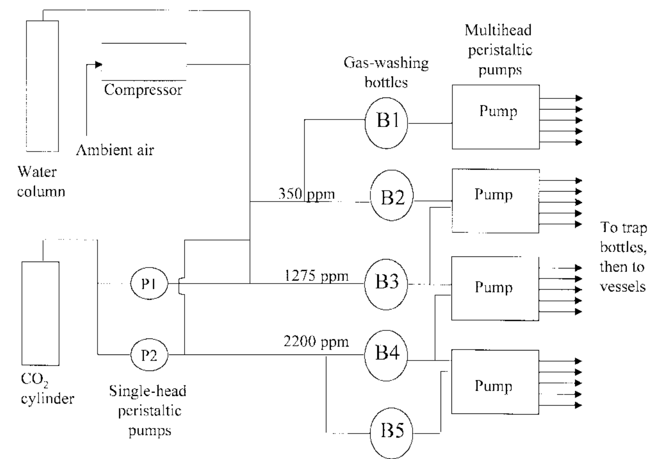

Figure 1–Delivery of CO_2-enriched air to culture vessels.

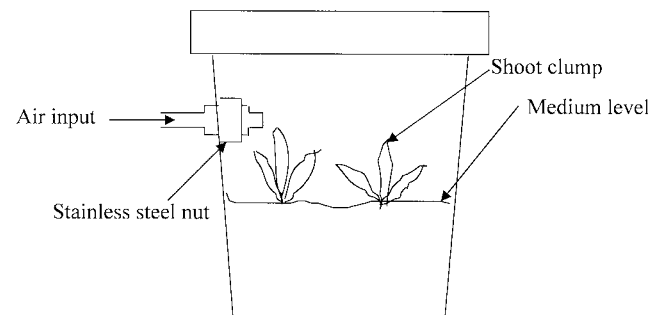

Figure 2–Ventilated culture vessel.

Statistical Methods

Statistical analysis of the data was done using Minitab software version 11. A fractional factorial design, rather than a full factorial design was applied because of the space limitations of the growth environment. According to the theory of the design, the maximum and minimum level for each factor constituted the corner points of the box and the middle value of each factor formed the center point (fig. 3) for a total of nine treatments ($2^3 + 1$). A full factorial design would have required all combinations of all treatment factors for a total of 27 treatments (3^3).

Statistical analysis was used to determine whether the factor effects were significant or not. Response surfaces were calculated and drawn using contour plots to analyze the response of shoots to the different treatment factors in order to find the optimum combination of treatment levels.

Experimental Methods

Because of space limitations, four replicates were chosen for each observation at the corner points and six for the observation at the center point, for a total of 38 vessels ($2^3 \times 4 + 6 = 38$). The high and low levels for CO_2 concentration were chosen to be 2200 and 350 µmol mol^{-1} (ambient), respectively. The middle point was 1275 µmol mol^{-1}. The high level for the hormone concentration was chosen to be equal to the concentration of the hormones in the regular multiplication medium and the low was chosen to be 0.1 times of that value. The middle point for hormone concentration was 0.5. The lowest PPF was 100, the highest was 180 and the middle point was 140 µmol m^{-2} s^{-1}. All vessels were placed on the shelf in the growth chamber. Randomization was achieved for CO_2 and hormone treatments (but not light level) to avoid biasing with respect to the location of the vessel in the growth chamber.

The method used to allocate shoots to the vessels ensured that all treatments had equal initial wet weights. Shoots from each stock vessel were cut into clumps of an average size of four or five shoots each, under an aseptic hood. For each treatment vessel, four to five of these shoot clumps were then grouped on a sterile paper towel and weighed. All the groups had the same target weight and if the weight of a group was too high or too low, adjustments were made either by replacing a clump with a larger or smaller clump or adding more shoots to the group. Adjustments led to a small range of variation for the total weight of shoots going to each vessel. Neither the target nor the individual group weights were permanently recorded, but since all groups initially weighed the same and since transformation (survival and not growth *per se*) was the objective of the study, this oversight is not seen as critical. Finally, a treatment was randomly assigned to each vessel to reduce any treatment bias caused by number of shoots per vessel or other factors. The group of shoots from each vessel was destructively analyzed for dry weight at the end of the experiment.

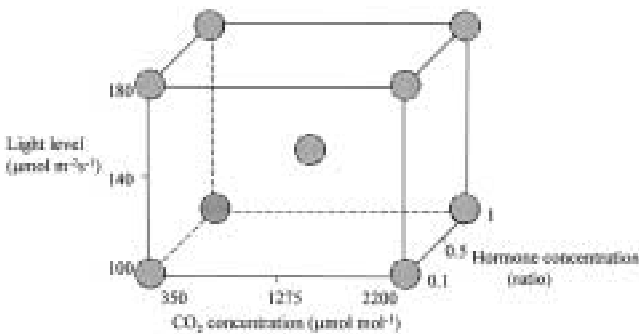

Figure 3–Schematic for the factorial design. The points show the nine treatment combinations.

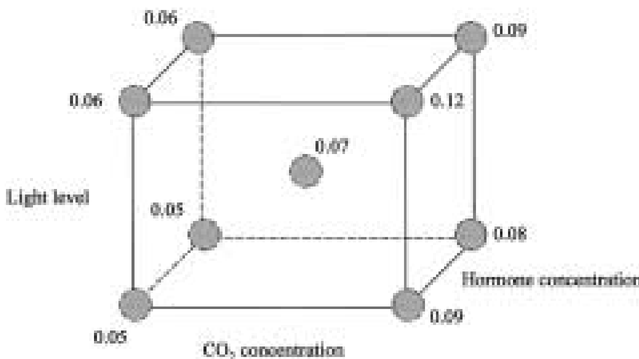

Figure 4–Average final dry weights of shoots per vessel (g) for treatment points shown in figure 3.

Results and Discussion

As a general observation from the experiment, the vessels treated with higher light and higher CO_2 concentrations had greener and more vigorous shoots. The data for the average final dry weight with respect to different factor levels are shown in table 1 and summarized in figure 4. Letters H, L, and M in table 1 represent the high, low, and middle level of each factor. The p-values from the statistical analysis of the dry weight data are given in table 2.

The largest final dry weight was obtained with a combination of high light level, high CO_2 concentration and low hormone concentration. The results in table 1 clearly show that the treatments with high light and high CO_2 concentration had larger average final dry weight than the treatments with low light and low CO_2 concentration. Observations of the color of the plants at the end of the experiment supported these data. All shoots in all vessels were observed to become yellowish in color and lose vigor in the sugarless environment at the beginning of the

Table 1. Average dry weights resulting for treatments

Hormone Concentration	Light Level (µmol m^{-2} s^{-1})	CO_2 Concentration (µmol mol^{-1})	Final Dry Weights (g)	Average Final Dry Weight (g)
H	H	H	0.09, 0.07, 0.12, 0.09	0.09
L	H	H	0.11, 0.12, 0.15, 0.11	0.12
H	L	H	0.06, 0.09, 0.08, 0.08	0.08
L	L	H	0.13, 0.06, 0.09, 0.09	0.09
H	H	L	0.06, 0.06, 0.07, 0.04	0.06
L	H	L	0.07, 0.07, 0.05, 0.05	0.06
H	L	L	0.04, 0.05, 0.07, 0.06	0.05
L	L	L	0.07, 0.06, 0.04, 0.05	0.05
M	M	M	0.07, 0.06, 0.08, 0.07 0.11, 0.05	0.07

Table 2. P-values for factors

Factors	p-Value
Hormone concentration	0.067
Light level	0.044
CO_2 concentration	0.000

experiment. However, the high light, high CO_2 treatments regained their green color and vigor indicating they successfully achieved photoautotrophy; whereas, the low light, low CO_2 plants remained pale and yellowish indicating they did not achieve photoautotrophy. There was not much of a visual difference in color between the low and medium conditions treatments.

The final dry weight was noted to decrease with increasing hormone concentrations. The average dry weight of the treatments for high light level, high CO_2 concentration and low hormone concentration was 0.12 g; whereas, the average dry weight of the treatments for high light intensity, high CO_2 concentration and high hormone concentration was 0.09 g.

The consistency of the data (fig. 4) adds a degree of confidence in the results. This consistency is seen by observing that the average final dry weight for the center treatment (0.07 g) is between the dry weights for diagonal corner treatments. This same trend is seen for each of the four possible diagonal corner combinations.

Regression analysis was used to test the significance of each factor. A p-value smaller than 0.05 is required at 95% level to show the factor as significant. The results (table 2) showed that the light level and CO_2 concentration effects were significant. The hormone concentration was not found to have a statistically significant effect on final dry weight.

Response surfaces were obtained from the data and the resulting contour plots are shown in figures 5a,b,c. The slope of these curves gives the per unit response to each treatment. The graphs did not show a region where maximum responses could be found. Apparently, the maximum was not achieved within the range of factor levels studied. The graphs confirmed that the response increased in the direction of increasing light level and CO_2 concentration but decreasing hormone concentration.

Summarizing, high light and high CO_2 concentration were found to be favorable factors for transformation of the shoots to photoautotrophy. These findings were in accordance with those of other studies such as Kozai and Iwanami (1988); Kozai et al. (1988); Kozai et al. (1990); Fujiwara et al. (1987); Cournac et al. (1992). The more the CO_2 and light are made available to the plants, the more the plants will utilize these for photosynthesis (unless the CO_2 and light compensation points are achieved) and the easier the transformation to photoautotrophy will occur. Even so, the positive effect of CO_2 concentration was somewhat surprising because sugarcane is a C4 plant.

The literature reveals that enhanced CO_2 concentrations in culture vessels might prevent some of the effects resulting from ethylene accumulation (Kumar et al., 1996). In some of their cultures with *Paulownia*, the retardation of plant growth in sealed vessels was thought to be due to the CO_2 deficiency. Thus, enriching the gas phase with CO_2 or reducing the partial pressure of O_2 reversed the inhibitory effect of ethylene on the process of greening of spinach cell cultures. It seems possible that in the current study CO_2 enrichment of the air inside the vessels caused a repression of the ethylene effect which in turn benefited transformation. However, the probability of this is reduced because CO_2 enrichment was achieved by continuously flowing CO_2 enriched air into the vessels which would have flushed out much of the ethylene generated by the plants.

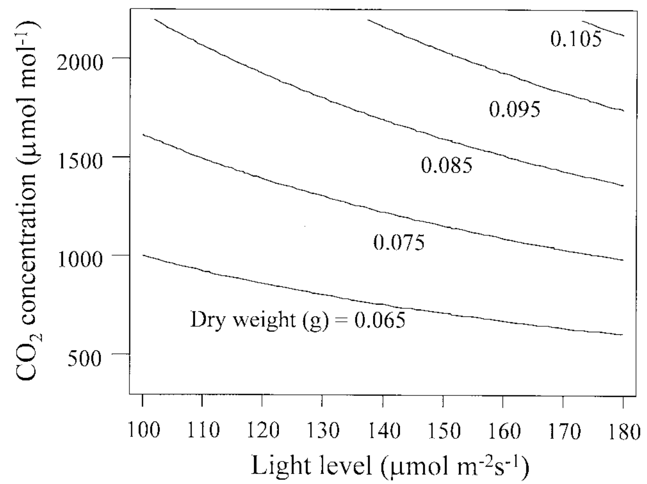

Figure 5(a)–Effects of CO_2 concentration and light level on the final dry weight.

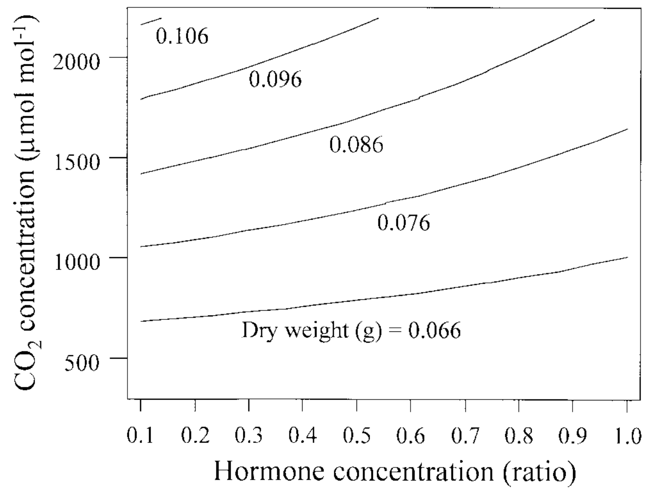

Figure 5(b)–Effects of CO_2 and hormone concentrations on the final dry weight.

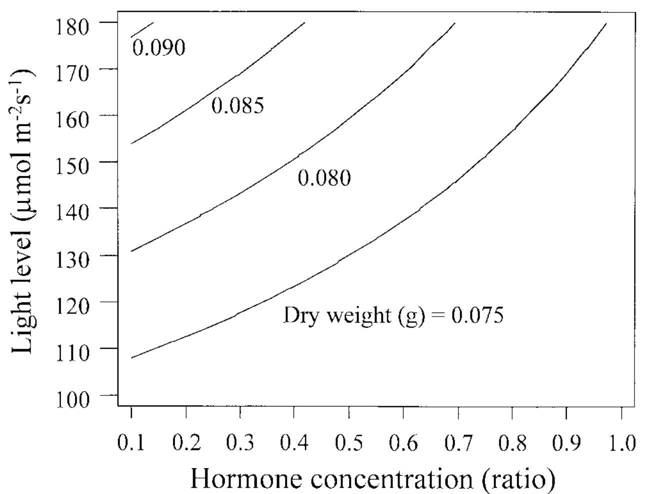

Figure 5(c)–Effects of light level and hormone concentration on the final dry weight.

CONCLUSION

Higher light level and higher CO_2 concentration stimulated the transformation of sugarcane shoots to photoautotrophy as indicated by final dry weight. The effects of light level and CO_2 concentration were found to be significant and positive while the effect of hormone concentration was negative but insignificant. It can be expected based on the findings of this study that the high levels for the two significant factors, i.e., a 2200 μmol mol^{-1} CO_2 concentration and 180 μmol m^{-2} s^{-1} light level, will stimulate the transformation to photoautotrophy in sugar-free medium of sugarcane shoots previously micropropagated in medium with sugar. It seems appropriate to keep the hormone concentration the same as is used in conventional micropropagation media with sugar.

Research can be extended in the future to find the exact optimum points for the effect of environmental factors for the shoots on transformation to photoautotrophy. The optimum region for the intensity of light and CO_2 concentrations can be found by extending the region used in this study in the direction of increased light levels and CO_2 concentrations. It should be noted that the current research only addresses the transformation step to photoautotrophy. While this is an important first step, further research is required to determine the optimal conditions for multiplication of photoautotrophic sugarcane shoots and to determine whether the decreased costs of working in septic conditions lead to reduced cost per shoot produced.

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