Effect of 2-(3,4-Dichlorophenoxy)triethylamine on Guayule (*Parthenium argentatum*) Cell Suspension Cultures

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2-(3,4-Dichlorophenoxy)triethylamine (DCPTA) promoted both cell growth and rubber production in guayule (*Parthenium argentatum*) cell suspension cultures. Concentrations as low as 0.05 mg/L of DCPTA were found to be effective. Cell growth increased 3-fold over that of controls when treated with 0.75 mg/L DCPTA. Likewise, a 72% increase in rubber accumulation at 0.3 mg/L was observed. Optimal concentration was found to be 0.5 mg/L, which caused a total of 4-fold increase in rubber production. Chlorophyll biosynthesis was also enhanced.

INTRODUCTION

The rubber-producing shrub, guayule (*Parthenium argentatum* Gray), is the subject of intense commercialization research. Low rubber yields from conventional planting techniques have led to interest in plant tissue culture techniques for rubber production. In vitro rubber production was first demonstrated in cultured guayule stem segments in 1950 (Arreguin and Bonner, 1950). Rubber production was also demonstrated in guayule callus cultures (Radin et al., 1982).

The chemical bioregulator 2-(3,4-dichlorophenoxy)triethylamine (DCPTA) has been shown to stimulate rubber synthesis in both field-grown and greenhouse-grown guayule plants (Yokoyama et al., 1977). This could be attributed to the increased total activity of key enzymes in the isoprenoid biosynthetic pathway, such as mevalonic acid kinase and isopentenyl pyrophosphate isomerase, in DCPTA-treated guayule plants (Benedict et al., 1983). DCPTA was also found to produce either guayule shoot or callus tissue exclusive of each other, as a function of DCPTA concentration (Dastoor et al., 1981). DCPTA enhanced guayule callus growth, but it did not increase its rubber productivity. In shoot cultures, however, rubber production was increased but not growth (Staba and Pagana, 1988). The purpose of this research was to study the effect of various concentrations of DCPTA on the cell growth and rubber production of guayule cell suspension cultures.

MATERIALS AND METHODS

Cell Line. Selected polyploid cell suspension cultures were a gift from Professor T. Murashige of the University of California at Riverside, Riverside, CA.

Culture. Cultures were maintained in a medium composed of the usual MS salt mixtures (Murashige and Skoog, 1962) with the following modifications: NH_4NO_3 14.67 mM, $Ca(NO_3)_2 \cdot 4H_2O$ 6 mM, NaFeEDTA 0.01 mM, thiamin hydrochloride 1 mg/L, glycine 2 mg/L, picloram (4-amino-3,5,6-trichloropicolinic acid from Dow Chemical Co.) 0.03 mg/L, benzyladenine 0.01 mg/L, sucrose 30 g/L, and inositol 100 mg/L. An inoculum of ca. 300 mg fresh weight of each was added to 25 mL of the above medium in 125-mL De Long flasks. Cultures were grown on a gyratory shaker (150 rpm) at 27 °C under a 16-h light/8-h dark cycle. Light intensity was maintained at 1000 lux provided by Gro Lux lamps. Each treatment group consisted of eight flasks, and all of the flasks were randomly arranged on the shaker. At the end of the 4-week experimental period, replicate flasks were randomly grouped into four subgroups of two to be used as analysis replicates.

Rubber and Chlorophyll Analysis. Cell cultures were harvested by filtration, lyophilized, and extracted with acetone and methylene chloride for 8 and 20 h in a Soxhlet apparatus, respectively. The methylene chloride fraction was evaporated under vacuum. The residue (rubber) thus obtained was purified by adding warm acetone. After cooling, the precipitate was washed with cold acetone. The presence of rubber was verified by ^{13}C NMR (Hayman et al., 1982), and the yield of rubber was determined by weighing the purified sample. Combined acetone fractions were used for determining the total chlorophyll. Chlorophylls were quantified spectrophotometrically according to the published equation (Lichtenthaler, 1987).

Chemicals. 2-(3,4-Dichlorophenoxy)triethylamine (DCPTA) was synthesized from (*N*,*N*-diethylamino)ethyl chloride hydrochloride and 3,4-dichlorophenol according to the published method (Schuetz and Baldwin, 1958). DCPTA was filter-sterilized prior to being added to the autoclaved culture medium. Picloram was a gift from Dow Chemical Co.

Statistical Analysis. Analysis of variance (ANOVA) and Dunnet's test for comparing the mean of control to the mean of each treated group were performed for all of the data obtained in this study; the data were also subjected to linear and polynomial regression analyses to find the best fit model for the data (Zar, 1974).

RESULTS AND DISCUSSION

Effect on Cell Growth. Guayule cell suspension cultures were maintained for ca. 1 year prior to this study. Cell suspension cultures grew as compact 1–3 mm diameter green cell aggregates. No browning of tissues was observed during the 4-week experimental period. The medium was replaced once at 2 weeks. Staba and Pagana had shown that 2 mg/L DCPTA increased guayule callus growth and that 20 mg/L DCPTA inhibited it; they also found that DCPTA inhibited guayule shoot culture growth at concentrations 2 mg/L and above (Staba and Pagana, 1988). In this study, it was found that the effect of DCPTA is concentration dependent. The stimulatory effect on cell growth increased as the concentration of DCPTA increased between 0.05 and 2 mg/L (Table I). A maximum of 3-fold increase of cell growth over the control cultures was obtained at 0.75 mg/L DCPTA. At greater concentrations of DCPTA, the stimulatory effect started to decline. At 10 mg/L DCPTA, 20% inhibition of cell growth was observed (Table I).

Effect on Rubber Production. It was reported that light-grown guayule callus cultures produced about 10 times the amount of rubber over dark-grown cultures (6.0 vs 0.5 mg/g of dry wt) (Radin et al., 1982). In this study cell suspension cultures were grown under a 16-h light/8-h

Table I. Effect of DCPTA on Guayule (*P. argentatum*) Cell Suspension Cultures

treatment, mg/L	growth index[a]	× control	rubber content mg/g of dry wt	× control	total rubber prod,[e] × control	total chlorophyll μg/g of dry wt	× control
control	23.51 ● 2.58[b]	1	14.1 ± 1.15	1	1	183.7 ± 9.11	1
DCPTA, 0.05	30.15 ± 3.34 *[d]	1.28	17.2 ± 1.73 *	1.22	1.56	213.0 ± 8.50 **	1.16
DCPTA, 0.10	41.08 ± 3.10 **	1.75	18.7 ± 2.43 **	1.33	2.33	212.7 ± 6.36 **	1.16
DCPTA, 0.30	53.98 ± 5.18 **	2.30	24.3 ± 2.07 **	1.72	3.96	234.8 ± 7.62 **	1.28
DCPTA, 0.50	69.07 ± 5.26 **	2.94	19.4 ± 2.17 **	1.38	4.06	292.3 ± 12.34 **	1.59
DCPTA, 0.75	72.13 ± 4.03 **	3.07	15.1 ± 2.02 NS	1.07	3.29	282.8 ± 11.28 **	1.54
DCPTA, 1	56.69 ± 4.51 **	2.41	13.8 ± 1.41 *	0.98	2.36	243.6 ± 8.02 **	1.33
DCPTA, 2	42.67 ± 5.11 **	1.82	8.8 ± 1.74 **	0.62	1.13	230.6 ± 4.80 **	1.26
DCPTA, 5	24.50 ± 5.37 NS	1.04	6.5 ± 1.53 **	0.46	0.48	193.4 ± 9.22 NS	1.05
DCPTA, 10	18.30 ± 2.82 *	0.78	6.2 ± 1.46 **	0.44	0.34	188.9 ± 9.89 NS	1.03
ANOVA[c]	**		**			**	
linear[c]	**		**			**	
quadratic[c]	**		**			**	

[a] Growth index = net growth g of fresh wt/g of inoculum. [b] Each recorded value represents the mean ± standard deviation of four determinations. Data were subjected to general linear model T test for the null hypothesis against control. [c] Data were subjected to ANOVA, linear, and polynomial regression analyses. [d] NS, *, **, nonsignificant, significant at the 5% and <1% levels, respectively. [e] This column represents the total rubber production change of the treated cultures as compared to control cultures. It is obtained by multiplying the growth change factor and rubber content change factor, e.g., in the case of 0.5 mg/L DCPTA, 2.94 × 1.38 = 4.06.

dark cycle. Rubber content of 14.1 mg/g of dry wt was found in control guayule cell suspension cultures under this experimental condition. This represents a 2.35-fold increase of rubber production over that of the callus culture mentioned above, making the guayule cell suspension culture a better rubber-producing source. Table I shows the concentration-dependent rise in enhancement of rubber production in cell cultures treated with lower concentrations of DCPTA: 0.05 mg/L DCPTA caused a 22% increase. Increasing concentrations of DCPTA increased rubber production in a dose–response manner. A maximum stimulation of 72% over the control culture was obtained with 0.3 mg/L DCPTA. The stimulatory effect of DCPTA lessened above this concentration. A slight inhibition of rubber synthesis was observed at 2 mg/L DCPTA and a 60% inhibition at 10 mg/L DCPTA. These results are analogous to those of guayule shoot culture: a low concentration of DCPTA (2 mg/L) increased rubber production, while a higher concentration (20 mg/L) inhibited it (Staba and Pagana, 1988).

Effect on Chlorophyll Content. A steady rise in the total chlorophyll content was observed as the concentration of DCPTA increased to 0.5–0.75 mg/L (Table I). Use of greater DCPTA concentrations caused chlorophyll content to decline. The stimulatory effect of DCPTA diminished at 5 mg/L and changed to a 30% inhibition at 20 mg/L. The effect of DCPTA in this case is similar to the one we observed in the green alga *Euglena gracilis* (Hsu and Yokoyama, 1986). We suggested that increased synthesis of total chlorophyll in turn caused stimulation of total photosynthesis. The effect of DCPTA on chlorophyll and photosynthesis should result in increased flow and utilization of photosynthetic carbon in the biosynthetic and metabolic pathways. Thus, the effect of DCPTA on total chlorophyll content in cell culture could be a possible explanation of the role of DCPTA in promoting the cell growth and the synthesis of *cis*-polyisoprene (rubber) in guayule cell suspension cultures. Since the cell growth peaked at 0.75 mg/L DCPTA, yet the rubber content peaked at 0.3 mg/L, we suggest that DCPTA affects cell growth and channelling of photosynthate to rubber production differently. Therefore, factors other than enhanced photosynthesis or increase in carbon source may also be involved in affecting the rubber production. Through its effect on cell growth and rubber-producing capacity, DCPTA (0.5 mg/L) was found to be the optimal concentration; it caused a total of 4-fold increase in rubber production over that of the control. Although the greater rubber yields obtainable through DCPTA-treated cultures are not quite at the level of intact plant (24 mg/g of dry cell wt vs 80–260 mg/g of dry intact plant) (Vietmeyer, 1977), the cell culture system might still be commercially significant due to its shorter culturing time required for rubber production and the ease of sample harvest. Information gained about the nature of the concentration dependence of the DCPTA response in this study may also be applicable to field applications or to a newly designed bioregulator.

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LITERATURE CITED

Arreguin, B.; Bonner, J. The biochemistry of rubber formation in the guayule. II. Rubber formation in aseptic tissue cultures. *Arch. Biochem.* **1950**, *26*, 178–186.

Benedict, C. R.; Reibach, P. H.; Madhavan, S.; Stipanovic, R. V.; Keithly, J. H.; Yokoyama, H. Effect of 2-(3,4-dichlorophenoxy)-triethylamine on synthesis of cis-polyisoprene in guayule plants (*Parthenium argentatum* Gray). *Plant Physiol.* **1983**, *72*, 897–899.

Dastoor, M. N.; Schubert, W. W.; Peterson, G. R. Preliminary results of in vitro propagation of guayule. *J. Agric. Food Chem.* **1981**, *29*, 686–688.

Hayman, E.; Yokoyama, H.; Schuster, R. Carbon-13 nuclear magnetic resonance determination of rubber in Guayule (*Parthenium argentatum*). *J. Agric. Food Chem.* **1982**, *30*, 399–401.

Hsu, W.-J.; Yokoyama, H. Effect of 2-(3,4-dichlorophenoxy)-triethylamine (DCPTA) on the photosynthetic capacity of *Euglena gracilis* Z. *Abstracts of Papers*, 192nd National Meeting of the American Chemical Society, Anaheim, CA; American Chemical Society: Washington, DC, 1986; Abstract AGFD 45.

Lichtenthaler, H. K. Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, *148*, 350–382.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 473–497.

Radin, D. N.; Behl, H. M.; Prokisch, P.; Rodriguez, E. Rubber and other hydrocarbons produced in tissue cultures of guayule (*Parthenium argentatum*). *Plant Sci. Lett.* **1982**, *26*, 301–310.

Schuetz, R. D.; Baldwin, R. A. The synthesis and properties of some substituted phenyl-ω-(N,N-dialkylamine)-alkyl sulfides. *J. Am. Chem. Soc.* **1958**, *80*, 162–164.

Staba, E. J.; Pagana, S. A. Growth regulator effects on rubber and resin production in guayule callus and proliferating shoot cultures. *J. Nat. Rubber Res.* **1988**, *3*, 127–135.

Vietmeyer, N. D. *Guayule: An Alternative Source of Natural Rubber*; National Academy of Sciences, Washington, DC, 1977.

Yokoyama, H.; Hayman, E. P.; Hsu, W.-J.; Poling, S. M.; Bauman, A. J. Chemical bioinduction of rubber in guayule plant. *Science* **1977**, *197*, 1076–1077.

Zar, J. H. *Biostatistical Analysis*; Prentice-Hall: Englewood Cliffs, NJ, 1974.

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