EFFECTS OF 2-CHLOROETHYLPHOSPHONIC ACID (ETHEPHON)

ON SCENEDESMUS QUADRICAUDA

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The effects of various concentrations of 2-chloroethylphosphonic acid (Ethephon), an ethylene-releasing compound, on the total protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) levels in Scenedesmus quadricauda IU 614 were investigated.

The alga was grown under 450±50 ft-c continuous light illumination at 24±1 C with varying concentrations of Ethephon (0.0, 0.00001, 0.0001, 0.001, 0.1, 1.0, and 10.0 µg/ml). The nucleic acids were extracted and quantitated by the diphenylamine and orcinol methods. Protein was determined by the Lowery method.

Total protein and DNA levels were not significantly different from those in controls. The RNA level was increased by 26% over control when the alga was treated with 0.1 µg/ml Ethephon. Ethephon, at concentrations of 0.1 and 1.0 µg/ml, stimulated growth significantly above controls. The other concentrations of Ethephon did not stimulate growth above the control cultures.

EFFECTS OF 2-CHLOROETHYLPHOSPHONIC ACID (ETHEPHON) ON *SCENEDESMUS QUADRICAUDA*

THESIS

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EFFECTS OF 2-CHLOROETHYLPHOSPHONIC ACID (ETHEPHON) ON SCENEDESMUS QUADRICAUDA

The discovery that 2-chloroethylphosphonic acid (Ethephon) decomposes at physiological pH to release ethylene has led to its use as a method of introducing exogenous ethylene into plant tissues (4). Ethephon has been found to affect a number of physiological processes in plants, including the alteration of apical dominance, stimulation of lateral bud growth, acceleration of abscission of fruit, acceleration of fruit ripening, and induction of lateral root formation. While there is an abundance of literature citing the effects of ethylene-releasing compounds on higher plants, little is known about the effects of ethylene on algae (1,6, and 15). The algae cannot be treated as physiological duplicates of higher plants (13). For instance, in 1970, Wagner (18) reported that the diatom, Nitzschia putrida, showed an increase in cell division when exposed to an atmosphere containing ethylene, while Adelbaum and Burg (3) reported that ethylene inhibited cell division in Pisum sativum. Indeed, Wagner's work appears to be the only one published on the effects of ethylene on aglae.

Therefore, it was the purpose of this study to investigate the effect of various concentrations of Ethephon on the total protein content, deoxyribonucleic acid (DNA) content, ribonucleic acid (RNA) content, and growth of the Chlorophyceaen alga, Scenedesmus quadriacauda.

MATERIALS AND METHODS

Organism and Culture Conditions

Stock cultures of Scenedesmus quadricauda IU 614 were maintained on Bristol's agar as modified by Bold (5). Fifty 250-ml Erlenmeyer flasks, containing 48 ml of Bold's modified medium and stoppered with synthetic sponge plugs, were prepared and autoclaved for 18 min at 121 C and 17 psi, cooled, and inoculated with a stock culture to a final optical density (O.D.) of 0.04 at 745 nm. The flasks were placed on an Eberbach reciprocal shaker (modified to hold 50 flasks) and agitated at 80 cycles per min (reciprocal stroke, 3.5 cm) under a continuous illumination of 450±50 ft-c of light as provided by 40-watt cool-white fluorescent tubes in a Sherer Gro-Lab, Model CEL 25-7, chamber.

Ethephon 68-241, supplied courtesy of Amchem Products, was recrystallized 3 times from benzene to yield a fraction with a melting point of 74 C. The fifty flasks were divided into 10 groups for treatment. At the time of inoculation, Ethephon was added to the various groups of flasks to a final concentration of 0.0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1.0, and 10.0 µg/ml. Five flasks were not inoculated and served as blanks. Each treatment was applied to 5 flasks, and this part of the study was repeated twice.

The cultures were grown for 8 days and the O.D. at 745 nm was determined daily. A Coleman-Hitachi Double Beam Spectrophotometer, Model 124, set on scale 1, slit 1 nm, was used to

determine O.D. in this study. The pH was determined daily with a Leeds-Northrup pH meter.

For the second part of the study, 10 l of Bristol's modified medium were prepared, placed in two 5-l fermentor flasks, and autoclaved for 1 hr at 121 C and 17 psi, cooled, and inoculated with 500 ml of a stock culture of S. quadricauda. One fermentor flask was treated with 0.1 µg/ml Ethephon and the other fermentor flask served as a control. These flasks were placed in a New Brunswick MicroFerm Fermentor, Model MF-207F, equipped with light manifolds, with the agitation set at 150 rpm, the temperature at 30 C, the input air pressure at 14.7 psi, and the sterile air flow at 1000 ml per min. These cultures were grown 7 days and then harvested. This part of the study was repeated twice, and the positions of the treated and control flask were reversed for the second run.

Cell Numbers

Coenobia numbers were determined microscopically with the aid of an AO Spencer Bright-line Haemocytometer. The number of coenobia per ml was correlated with the O.D. (745 nm) and a nomogram was prepared to permit the conversion of O.D. to coenobia/ml (Fig. 1). Multiplying the O.D. by 2.4 (the slope of the standard curve) gave the approximate coenobia/ml x 10^6.

Preparation of Cells for Biochemical Tests

After 7 days, the cells from each 5-l batch culture were harvested by filtration and washed twice with Tris buffer (pH 7.1). The cells were then frozen in an acetone-ice mixture and stored at -5 C until used.

The cells were rapidly thawed in a 37 C water bath when used. The total volume of cell suspension was brought to 50 ml with Tris buffer (pH 7.1); 10 ml of this suspension were used for the dry weight determination. The cells were disrupted in a Braun Cell Homogenizer, Type 853033, using 0.4 ml of 0.15±.05 mm Glasperlen glass beads per ml of cell suspension. This treatment resulted in the disruption of 95 per cent of the cells. The glass beads and debris were removed by centrifugation at 300 g for 3 min. The supernatant was decanted and mixed with 20 ml of cold (5 C) 10% trichloroacetic acid (TCA) and retained in a cold room (5 C) for 10 min, after which the suspension was centrifuged for 5 min at 1000 g and the pellet, containing the nucleic acids and proteins, was removed and saved. The pellet was treated with 10 ml of 5% TCA, heated in a water bath (95-100 C) for 10 min, then immediately centrifuged at 1000 g for 3 min. The supernatant was decanted and saved. The residue was again treated with 10 ml of 5% TCA and heated in a water bath for 5 min, centrifuged 3 min at 1000 g, and the supernatant decanted and added to the supernatant collected previously for the nucleic acid determinations.

After cooling to room temperature, this solution was used for the protein determination.

Dry Weight Determination

Ten ml of cell suspension were divided into 5 two-ml aliquots, placed in tared crucibles and dried in an oven at 100-105 C. After 24 hrs, the crucibles were cooled to room temperature in a desiccator and weighed to determine the dry weight.

Ribonucleic Acid Determination

Using the orcinol method as modified by Clark (8), 3.0 ml of the solution (the supernatant separated and retained for the nucleic acid determination) were placed in a test tube to which were added 6.0 ml of the orcinol-acid reagent (2 ml of 10% ferric chloride hexahydrate in 400 ml of concentrated HCl), followed by 0.4 ml of the alcohol-orcinol reagent (6.0 g of orcinol in 94.0 g ethanol). A blank was prepared as above, substituting 3.0 ml of distilled water for the algal fraction. The solutions were mixed on a Lab-Line vortex mixer and heated in a water bath (95-100 C) for 20 min. After cooling to room temperature, the O.D. (660 nm) was determined, using the blank to establish zero O.D. RNA samples of known concentration were prepared with Yeast RNA, Type XI (Sigma, R 6750), and used to make a nomogram for RNA determinations (Fig. 2). The readings were then converted to μg/mg, dry weight. Multiplying the O.D.

(660 nm) by 89.3 (the slope of the standard curve) gave the approximate RNA concentration.

Deoxyribonucleic Acid Determination

The diphenylamine test as modified by Clark (8) for the deoxypentose of DNA, was used to assay for DNA. A 1.0-ml aliquot of the nucleic acid fraction was combined with 2.0 ml distilled water. A blank was prepared, using 3.0 ml of distilled water. To each of these tubes was added 6.0 ml of the DNA reagent (4.0 g diphenylamine, 11 ml concentrated H_2SO_4, and 400 ml glacial acetic acid). The tubes were heated in a water bath (95-100 C) for 20 min, cooled to room temperature, and the O.D. at 600 nm was determined, using the blank to obtain zero O.D. Known concentrations of calf thymus DNA, Type I (Sigma, D 1501), were used to make a nomogram (Fig. 3). Multiplying the O.D. (600 nm) by 1667 (the slope of the standard curve) gave the approximate DNA concentration.

Total Protein Determination

Using the Lowery method (12), a 0.5-ml aliquot of the basic protein solution was combined with 5.0 ml of reagent C (50 ml of 2% Na_2CO_3 in 0.1N NaOH, 0.5 ml of 2% potassium tartrate and 0.5 ml of 1% $CuSO_4$, mixed fresh before use), mixed well, and allowed to stand for 10 min. Folin-Phenol reagent was diluted 1:1 with distilled water, and 0.5 ml of this reagent was added to each tube, followed by rapid and complete

mixing on a Lab-Line vortex mixer. A blank was prepared as above, substituting 0.5 ml of distilled water for the basic protein solution. Known concentrations of Bovine Albumin (Pentex, BV-0162) were used to prepare a standard so that O.D. could be converted to µg/ml and then to µg/mg dry weight (Fig. 4). Multiplying the O.D. at 750 nm by 685 (the slope of the standard curve) gave the approximate total protein concentration.

Statistical Analyses

The daily growth data were analyzed statistically, using a one-way analysis of variance, after Steel and Torrie (17). Duncan's (9) Multiple Range Test was used to determine significance among treatments.

The data from the biochemical tests were analyzed statistically, using the least significant difference test (lsd) to determine if significant differences existed between the treated and the control cultures (17).

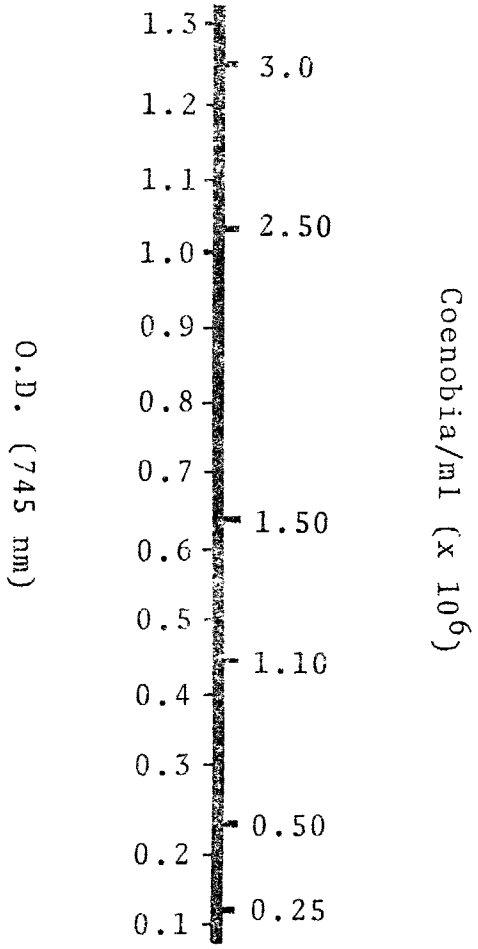

Fig. 1. Nomogram showing rectilinear relationship of O.D. (745 nm) to coenobia/ml x 10^6 for Scenedesmus quadricauda.

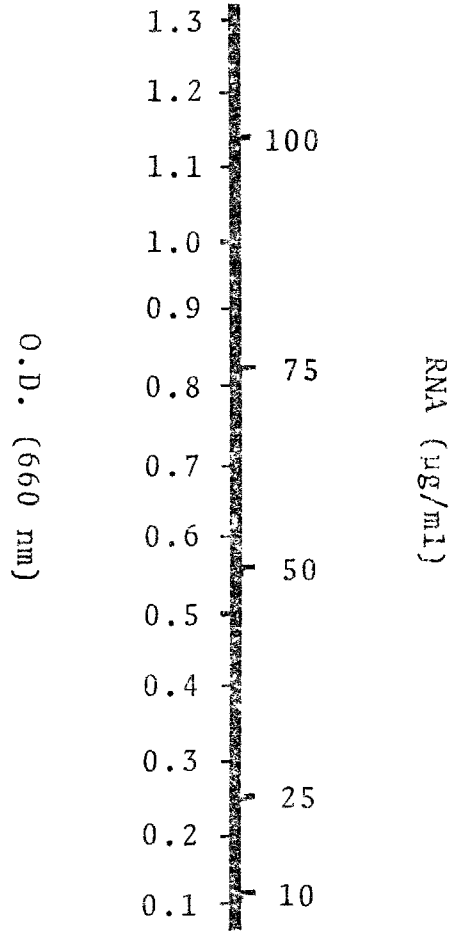

Fig. 2. Nomogram showing rectilinear relationship of O.D. (660 nm) to RNA standard (Yeast Type XI).

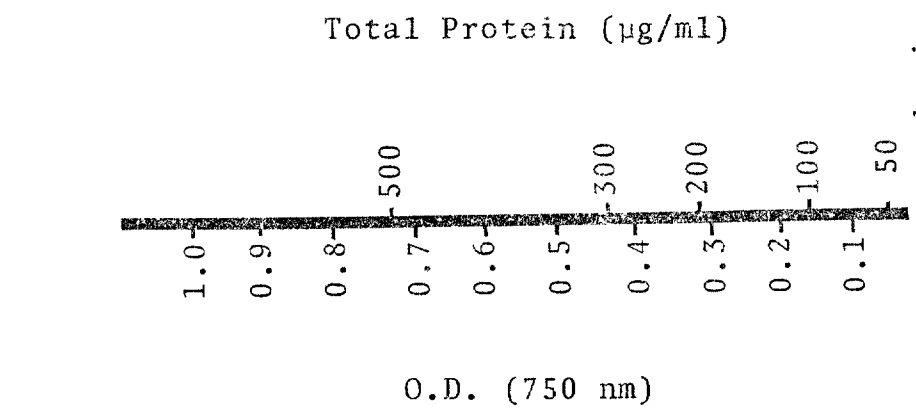

Fig. 4. Nomogram showing rectilinear relationship of O.D. (750 nm) to Protein standard (Bovine Albumin).

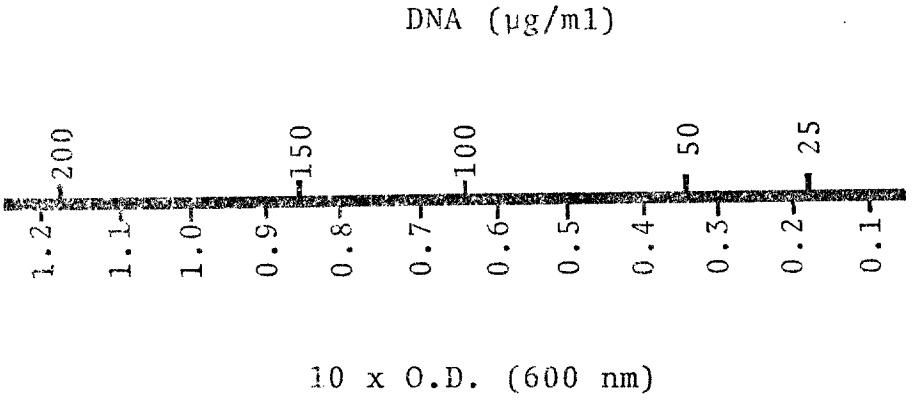

Fig. 3. Nomogram showing rectilinear relationship of O.D. (600 nm) to DNA standard (Calf Thymus, Type I).

RESULTS

An analysis of variance was performed on the growth data, and Table 1 shows the day 4, 5, 6, 7 and 8 means and their statistical significance. Day 1, 2, and 3 showed no significant differences. At no time was a significant difference in pH evident among treated and control cultures.

Concentrations of 1.0 µg/ml and 0.1 µg/ml resulted in a significant increase in growth of S. quadricauda and are shown in Fig. 5, while other concentrations tested did not produce a significant difference in growth. On day 8, there was a difference between the 0.1 µg/ml concentration culture and control culture of 7.68×10^5 coenobia/ml (3.07×10^6 cells/ml)

No significant differences existed in total protein or DNA levels between treatment and control cultures.

Significant differences were found between the RNA levels of control and treatment cultures (Table 2). In each case tested, Ethephon treatment resulted in increased RNA levels. The analysis of variance (ANOV) is presented in Table 3.

TABLE 1. Daily growth means (O.D. 745 nm) of Ethephon-treated cultures of Scenedesmus quadricauda. Each value is a mean of 5 replicates.

	ETHEPHON TREATMENT (µg/ml)						
Day	10.0	1.0	0.1	0.01	0.001	0.0001	Control (0)
4	0.21	0.23*	0.25	0.21*	0.20	0.18	0.18
5	0.38	0.43*	0.46*	0.39*	0.40*	0.34	0.34
6	0.71	0.82*	0.91*	0.78*	0.72*	0.68	0.65
7	0.98	1.14*	1.14*	1.02*	0.94*	0.92	0.89
8	1.23	1.44*	1.39*	1.29*	1.31*	1.21	1.13

* Significantly above control, 95% level, using Duncan's test.

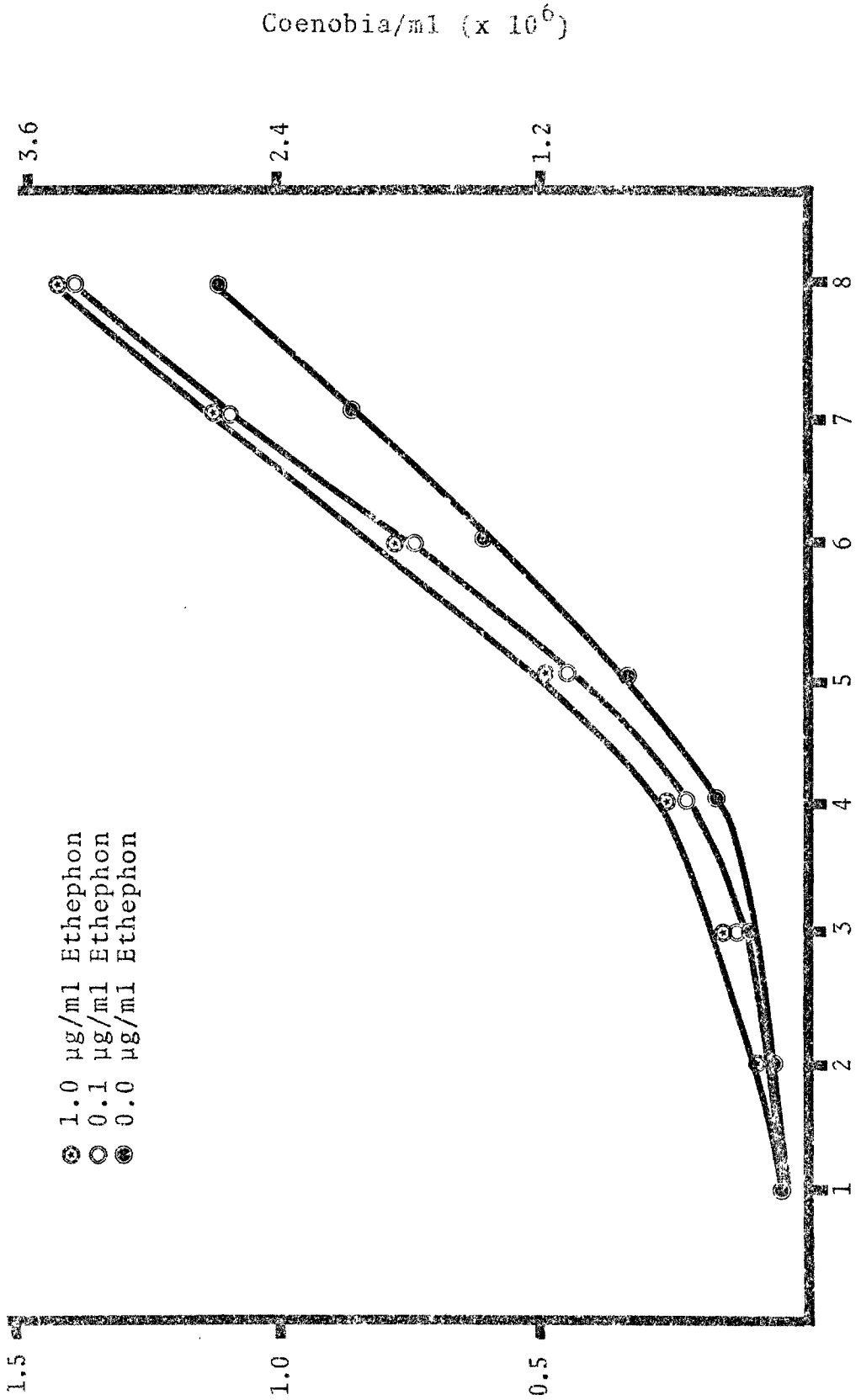

Fig. 5. Optical density and coenobia/ml (x 10^6) plotted against time for Scenedesmus quadricauda grown in various concentrations of Ethephon.

TABLE 2. RNA concentrations and significance for Ethephon-treated and control cultures of Scenedesmus quadricauda. Each value is a mean of five replicates.

	RNA CONCENTRATIONS (µg/mg of dry weight)	
RUN	TREATMENT	CONTROL
I	249.8*	188.2
II	252.3*	183.5
POOLED MEAN	251.1*	185.8

* Significant difference, 95% level, using the lsd test.

TABLE 3. Analysis of variance for significance of RNA levels of Ethephon-treated and control cultures of Scenedesmus quadricauda.

RUN	SOURCE OF VARIATION	df	SS	MS	F
I	TREATMENT	1	9,504.9	9,504.9	
	ERROR	8	5,694.4	711.8	13.55*
	TOTAL	9	15,199.3		
II	TREATMENT	1	11,819.8	11,819.8	130.24*
	ERROR	8	725.9	90.75	
	TOTAL	9	12,545.8		
POOLED	TREATMENT	1	21,261.7	21,261.7	58.97*
	ERROR	18	6,489.5	365.53	
	TOTAL	19	27,751.2		

* Significant difference at 95% level, using the F test.

DISCUSSION

Microscopic observation confirmed that the increased O.D. of the Ethephon-treated S. quadricauda cultures was due to an increase in the coenobia/ml and not increased cell size. Wagner (18) also reported increased cell division in the diatom Nitzschia putrida, when exposed to unknown amounts of ethylene released from apples. Whether this stimulation was a direct result of ethylene or some other gaseous product from the apples is unclear. In higher plants ethylene stimulates epinastic growth of leaf petioles, increased growth in diameter of pea epicotyls (15), and soybean stems (11). Osborne and Mullins (14) postulated that ethylene acted by interfering with the carrier protein of auxins, but it is doubtful that ethylene affects S. quadricauda in this manner, because the 4-cell coenobium would probably not have an elaborate carrier protein system. It also seems unlikely that the stimulation is a result of the heterotrophic utilization of ethylene, because of the extremely small quantity of exogenous ethylene added. Bristol's medium is relatively high in both Cl^- and PO_4^{+4}, which are produced as decomposition products of Ethephon; hence these factors are not considered to have a significant effect on growth.

In higher plant tissues, ethylene has been reported to inhibit DNA synthesis (7) and to increase protein synthesis

(10), but neither of these phenomena were observed in this study. Whether these do not occur, or occur only at certain periods in the life cycle (and therefore are not detected in a non-synchronus batch culture) is unknown.

Ethephon was found to stimulate RNA levels above controls, and this stimulation has also been reported in higher plants. Abeles and Holm (2) reported a primary role of ethylene in abscission to be an increase in RNA synthesis which preceded enzyme synthesis. One can speculate that the S. quadricauda RNA levels could have been altered in two ways. The Ethephon could have inhibited the enzyme, ribonuclease, which degrades messenger RNA (mRNA) to mononucleotides, thus leading to an accumulation of mRNA in the cells. This effect would not necessarily be related to the life cycle of S. quadricauda and thus would be seen even in non-synchronus cultures. Another possibility would be stimulation of de novo synthesis of RNA. Shimokawa and Kasai (16) report that C^{14} -labeled ethylene is incorporated into the 4s fraction of RNA in Pharbitis nil following a 120-min exposure to an atmosphere containing 100 ppm C^{14}-ethylene. Work with isotopes would be necessary to elucidate the exact effect of Ethephon on the RNA level of S. quadricauda.

It is unknown if ethylene is found endogenously, but exogenous applications cause significantly increased growth about 4 days following treatments in S. quadricauda and significantly different RNA levels between control and Ethephon-treated cultures.

This paper appears to be the only quantitative work in this field, and it indicates that Ethephon (ethylene) may function in a hormonal role in algae as well as in higher plants.

SUMMARY

Exogenous applications of Ethephon 68-241 (2-chloroethylphosphonic acid) were applied to cultures of Scenedesmus quadricauda IU 614. The alga was grown under 450±50 ft-c of continuous illumination at 24 C. The growth of the alga was analyzed and the concentrations of 0.1 µg/ml and 1.0 µg/ml were found to stimulate growth significantly above control cultures. The 0.1 µg/ml concentration was found to stimulate ribonucleic acid (RNA) levels 26 per cent above control cultures. This stimulation was found to be statistically significant. The deoxyribonucleic acid (DNA) and total protein levels were not significantly different than control cultures.

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