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RESPONSE OF CHLAMYDOMONAS REINHARDTII
AND SEVERAL VEGETABLE CROPS TO TRIACONTANOL
AND A MESSENGER(S) ELICITED BY TRIACONTANOL

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Teresa E. Kish

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RESPONSE OF *CHLAMYDOMONAS REINHARDTII* AND SEVERAL VEGETABLE CROPS TO TRIACONTANOL AND A MESSENGER(S) ELICITED BY TRIACONTANOL

By

Teresa Elaine Kish

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ABSTRACT

RESPONSE OF CHLAMYDOMONAS REINHARDTII AND SEVERAL VEGETABLE CROPS TO TRIACONTANOL AND A MESSENGER(S) ELICITED BY TRIACONTANOL

By

Teresa Elaine Kish

Field studies were conducted to compare the yield response of vegetables to triacontanol (TRIA) and a chemical(s) that is elicited by TRIA which stimulates plant growth (TRIM). TRIA increased the number of bell peppers in the late harvests and the size of wheat seed when adequate soil moisture was available. Foliar applications of TRIM increased wheat seed size, muskmelon fruit size, and bell pepper number and size. Studies comparing the effects of TRIA and TRIM application on Chlamydomonas reinhardtii suggested that TRIA and TRIM increase growth by different physiological processes. TRIA and TRIM increased total chlorophyll an average of 18% and 64%, respectively after incubation for 16-24 hr. The primary effects of TRIA and TRIM were on cell division and chlorophyll synthesis, respectively. A single enrichment of Chlamydomonas cell suspensions with CO_2 enhanced the activity of TRIM, however TRIA did not stimulate growth without this CO_2 enrichment.

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INTRODUCTION

It was recently discovered that Triacontanol (TRIA) elicits production of a biologically active chemical messenger in plants (TRIM) (49). This messenger may be responsible for the increased growth produced in plants by exogenous application of TRIA. Since several environmental and cultural conditions affect plant response to TRIA (41), it was hypothesized that these factors would affect plant response to the TRIA-messenger in a similar fashion. The purpose of this research was to study optimum conditions for TRIM response under field and laboratory conditions, and to compare these optima with those for TRIA. The TRIA messenger was compared with TRIA in vegetable yield trials. The growth parameters affected by TRIA and the messenger were studied in the laboratory using Chlamydomonas reinhardtii cultures. Treatment and culture conditions were manipulated to observe the differential response of plants or cells to TRIA and the messenger under various conditions imposed.

LITERATURE REVIEW

Discovery of Biological Activity of TRIA

Triacontanol (TRIA) was first isolated from alfalfa (*Medicago sativa*) leaves in 1933 and identified as a 30-carbon straight-chain primary alcohol (11). The biological activity of this naturally occurring constituent of plant waxes was discovered in 1977 (49). Coarsely chopped alfalfa applied as a sidedressing to tomato (*Lycopersicon esculentum*), lettuce (*Lactuca sativa*), and cucumber (*Cucumus sativus*) plots increased yields (41). The active compound was identified as 1-triacontanol ($CH_3(CH_2)_{28}CH_2OH$) by gas-liquid chromatography-mass spectrometry (49). TRIA is one of many long-chain alcohols present in the epicuticular waxes of leaves (13). Kolker (26) found it to occur as the free alcohol in cuticles, roots, leaves, and tissues in several plant species and in soil samples. Plants having a morphology that allowed sub-epidermal tissue to be sampled free of cuticular wax contamination were examined for the presence of TRIA beneath the epidermis. Jade (*Crassula argenta*) leaves, potato (*Solanum tuberosum*) tubers, and apple (*Malus domestica*) fruits appeared to contain TRIA beneath the epidermis. This suggests that TRIA is synthesized in the plant not only as a constituent of the cuticle, but also as a deposit in the mesophyll. TRIA was also found to be present in the sap of maple (*Acer saccharum*) trees (22). Endogenous TRIA may

participate in regulation of certain physiological activities (41).

Formulations and Dose-Response

The plant growth regulating activity by exogenously applied TRIA is well documented (42). The various *in vivo* and *in vitro* bioassays used to test activity require that the TRIA, which exists as crystals, be formulated in a homogenous liquid compatible with the biological systems in which it is used. Since TRIA is almost insoluble in water (2.1×10^{-16} M), various emulsions and dispersions have been formulated (40). TRIA was first used as a chloroform/water/Tween 20 emulsion. A solution containing 1 mg TRIA/ml chloroform was combined with water (containing 0.1% w/v Tween 20) to produce concentrations of 10 to 1000 µg/l (49). The chloroform droplets were evaporated from the emulsion by warming it on a stirring plate. Welebir (55) later developed a formulation in which TRIA was dissolved in acetone, 1 mg/ml, and combined with 7.5 mM aqueous $CaCl_2$, pH 8.0, to yield a final acetone concentration of 2% (v/v). The original formulation was modified by varying the $CaCl_2$ concentration and pH, sonicating the TRIA-acetone solution, and by adding 1.0 µm napthalene acetic or indole acetic acid (54).

A TRIA coagulum was developed at the Jiang Xi Engineering College, Nanchang, People's Republic of China (PRC). It is prepared by combining TRIA, extracted from beeswax, with Chinese dispersants and solidifying agents to form a thick, creamy emulsion that is squeezed from a metal dispensing tube. The coagulum, containing 1% TRIA, is prepared for application on plants by heating in water to 90-95° C, stirring until clear, and then diluting with water to the desired

concentration (38).

TRIA was formulated as a colloidal dispersion by sonicating TRIA crystals in water with a dispersant, tallow alkyl sulfate (TAS), (27). Sterile stocks containing 1000 mg/l may be diluted with distilled water to the desired concentration. This formulation appears superior for two reasons: (a) essentially all of the TRIA is uniformly dispersed as smaller particles and (b) very little dispersant (1% of the TRIA content) is needed. A comparison of formulations showed the optimum TRIA concentration for the colloidal dispersion was 0.01-1.0 mg/l compared to 10-100 mg/l for the other formulations (27).

Corn (Zea mays) and rice (Oryza sativa) seedlings responded to the colloidal dispersion in a quadratic fashion when applied to the foliage at concentrations up to 0.1 µg TRIA/l. Higher concentrations had progressively less effect, and did not decrease growth compared to the control (27). Quadratic yield responses of crops treated with TRIA were also observed (42). Optimum concentrations varied with the crop and location of the experiment.

Factors Affecting Plant Response

The conditions under which plants are treated with TRIA affect response. Air temperature is especially critical. Lower night temperatures enhance response to TRIA (32). It was suggested that lower night temperatures decreased respiration, which in turn increased food reserves upon which TRIA may act. A subsequent study, however, showed that the response was not altered by cooler night temperatures (5). A study of rice and corn exposed to air temperatures of 15, 25, and 35° C one hr before TRIA application showed pretreatment with the

higher temperatures enhanced response, whereas temperature after TRIA treatment had no effect (47).

The water status of plants also influences response to TRIA. TRIA typically does not produce a favorable response in plants undergoing water stress (5). TRIA decreased the yield of non-irrigated field corn but increased the yield when the corn was irrigated (39).

The pH of the colloidal dispersion and the volume of spray applied had no effect on TRIA activity (48). Single applications of TRIA are as effective as multiple applications. Foliar sprays, soil drenches, and seed soaks with TRIA also increased growth and sometimes yield (43).

The effectiveness of TRIA was different depending on the time of day applications were made (47). Corn seedlings grown in the greenhouse under midday light intensity of 800 to 1000 $\mu Es^{-1}m^{-2}$ exhibited 77% more growth when sprayed with TRIA at 1700 hr compared to those sprayed at 0900 or 1300 hr. In growth chambers, where light intensity was 300 to 400 $\mu Es^{-1}m^{-2}$, the optimum time to spray was 1300 hr. Photoperiod and night temperatures were the same in these greenhouse and growth chamber experiments, but relative humidity may have differed. In field experiments, significant yield increases of winter wheat (Triticum aestivum) occurred when TRIA was sprayed at 1700 hr, but not when applied at 0900 or 1300 hr (47).

The stage of crop development also influenced the response to TRIA, but it was difficult to separate this parameter from other varying environmental effects (5). Leafy vegetables generally responded best when TRIA was applied during rapid vegetative growth.

Fruiting crops and grains responded best when applications were at anthesis (42).

Inconsistency of Response

There is strong evidence that TRIA increases plant growth and metabolic activity, however, the failure of TRIA to consistently produce favorable results has plagued many researchers (5, 32). Fewer than 50% of the field tests with TRIA conducted in the United States (U.S.) have resulted in significantly increased yields (40). Even under controlled conditions in greenhouse or growth chamber studies, results have not always been reproducible (5). In contraast, TRIA has consistently increased yields of field crops in the PRC (40). Several factors may contribute to the inconsistency of response in the U.S., and several explanations have been offered as to why they are not a problem in the PRC. One factor may be the difficulty of formulating TRIA (40). The water solubility of TRIA dictates that it be prepared as an emulsion using dispersants or organic solvents. Particles may be unevenly distributed and their size may vary in spray dispersions or suspensions. It was believed that the fine particle size and uniform particle distribution of colloidal dispersions would eliminate much of this variability. The colloidal dispersion has caused the rapid response by plants at lower concentrations than previous formulations, but the lack of consistency and reproducibility have continued (5, 47).

Certain contaminants that may be present in TRIA spray mixtures inhibit plant response. Other long-chain alcohols, particularly octacosanol, which may contaminate TRIA extracted from plants, inhibit the action of TRIA (23). Phthalate esters, common plasticizers in

polyvinylchloride (PVC) tubing, also inhibit the response of plants to TRIA. No dry weight increases occurred when TRIA dispersions that had been passed through PVC tubing were applied to corn plants, however passage of the dispersion through natural rubber did not inhibit activity (46). Sprayers used in the PRC are equipped with natural rubber rather than PVC tubing commonly used in American spray systems. PRC formulations may be less subject to inhibition by contaminants. Perhaps the cultural practices used or environments in which the crops are grown in the PRC impose upon the plant some optimum conditions for response to TRIA (40).

Effects of TRIA on Plants

TRIA affects several physiological processes in plants. It delays senescence, as measured by the oat leaf senescence bioassay (4, 12, 51). No effect has been observed on percent germination in many species tested (10, 19, 29, 51). Stimulation of lettuce seed germination by gibberellin was decreased by TRIA, but no effect of light-stimulated germination of lettuce was observed (29). TRIA was more effective in enhancing mobilization of photosynthates in rice than were GA_3, kinetin, or auxin. In addition, the chlorophyll content and area of rice leaves were increased by TRIA treatments (12). Leaf area and dry weight of rice seedlings were increased by TRIA treatment whether grown in light or dark (44). TRIA increased protein content and cell number in haploid tobacco callus (15). The activity of several enzymes has been increased by treatment with TRIA (18, 28). Increases in dry weight, leaf area, reducing sugars, free amino acids, soluble protein, and reduced nitrogen occurred in whole rice and corn

plants within 10 min of treatment (45). Apparent increases in total nitrogen of whole plants were measured within 1 hr (25).

The rapid dry weight response after foliar treatment with TRIA was studied in growth analyses with rice (42). Net assimilation rate increased within 1 day of treatment but was no different than the control 2 and 6 days after treatment. After the initial dry weight increase over the control, any subsequent increase was apparently the result of the initial increase and not of continued TRIA action. A similar rapid effect was observed on sweet potatoes (Ipomea batatas) in the field (8). The dry weight and nitrogen content of leaves were increased 14 hr after TRIA treatment; however, no increase was observed in yield, percent dry weight, or protein content of mature roots.

Yield Response

Much of the variation in response to TRIA is evident in field trials. In Korea, TRIA applied as a foliar spray increased the yield of Chinese cabbage (Brassica campestris) and Chinese radish (Raphanus acanthiformis) 83 and 108%, respectively (30). Seed treatment with TRIA increased tomato and pepper (Capsicum annuum) yields. This resulted from an increased number of fruit per per cluster rather than from increased weight of individual fruit (31). Seed treatments of several crops in the field in the U.S. did not increase yield, but did increase the dry weight of carrot (Daucus carota), cucumber, barley (Hordeum vulgare), sugar beet (Beta vulgaris), and tomato in the greenhouse. Foliar sprays of the chloroform/water/Tween 20 emulsion of TRIA in the field the same year resulted in increased yield of cucumber, radish (Raphanus sativus), sweet corn, carrot, dry beans

(Phaseolus vulgaris), and tomato (54). More recently, the colloidal dispersion of TRIA tested in the field as a foliar spray resulted in increased yields in about 40% of all field experiments conducted over a 3-yr period. Eleven of the 13 crops tested responded favorably (5). The yield increase was particularly large for muskmelons (Cucumus melo) (40%) and was primarily a result of an increase in the number of melons per plant. In another study using the chloroform/water/Tween 20 emulsion on muskmelons, no increase in growth, yield, or soluble solids was observed (7).

The variation in response when the colloidal dispersion was tested in the field indicates that a special set of circumstances is required for favorable results. Researchers have unsuccessfully attempted to define conditions under which plants would consistently respond to TRIA (5, 32). However, in these studies, yield increases appeared most likely when foliar sprays of the colloidal dispersion, made from highly pure TRIA were applied at 0.1 to 1.0 µg/l. Application should be during rapid vegetative growth or anthesis on warm, sunny days in late afternoon, when plants are recovering from midday water stress (5).

Effect of TRIA on Algae

Algal cultures of Chlamydomonas reinhardtii and Anacystis nidulans have been used to study the effects of TRIA (17). They provide a source of easily maintained, uniform plant material that may be bioassayed in a short time. The chlorophyll content increased when Chlamydomonas cultures were incubated for 4 days with TRIA in the nutrient medium, and cell number increasd after 5 days. Anacystis increased significantly in cell number, but not chlorophyll content.

It was suggested that cell size may have been affected by TRIA treatment. Chlamydomonas incubated with TRIA exhibited reduced O_2 inhibition of photosynthesis; Anacystis was unaffected.

The effect of TRIA on C. reinhardtii is rapid. Houtz (21) observed increased photosynthetic CO_2 assimilation in Chlamydomonas within one hr of treatment with TRIA. Increases in photosynthetic CO_2 assimilation were observed when cells were cultured with high CO_2 (5% in air), but not when cells were cultured with low CO_2 (air). This lack of response to TRIA was attributed to decreased binding affinity of TRIA particles to these cells cultured with low CO_2 (21). Increases in cell density and total chlorophyll were observed after 3 days incubation of Chlamydomonas cultures with TRIA. The increase in chlorophyll was apparently a result of increased cell density, since no increase in amount of chlorophyll per cell was observed (21). Higher concentrations of the colloidal dispersion of TRIA are needed to stimulate algae than are needed to stimulate growth of higher plants (21). Ca^{+2} and K^{+} ions in the Chlamydomonas growth medium apparently caused flocculation of the dispersion. An increase in particle size decreased the effectiveness of the formulation.

Physiological Responses

Attempts to show how exogenously applied TRIA moves into and within the plant have been unsuccessful (40). However, TRIA was shown to move in the vascular system of maple trees. Xylem exudate collected during spring sap flow contained physiologically active concentrations of TRIA (22).

Stimulation of growth in tomato (C_3), but not corn (C_4) led to

the suggestion that TRIA affects the balance between photosynthesis and photorespiration (14). This conclusion was substantiated by researchers who observed reduced sensitivity of photosynthetic CO_2 assimilation to O_2 in C. reinhardtii incubated with TRIA (17). Ries, et al. (40) however, observed increased growth of corn and other C_4 plants, and Houtz (20) did not detect reduced sensitivity of photosynthetic CO_2 assimilation to O_2 in C. reinhardtii cells incubated with TRIA. The TRIA induced increase in photosynthetic CO_2 assimilation measured was attributed to an increase in ribulose 1,5-bisphosphate levels.

The dark response to TRIA indicated it is not directly involved in photosynthesis (44), but that its initial effect may be on stored carbon (6). Carbon dioxide appears to play a regulatory role since the dark response occurs only in its presence (6). TRIA appears to initiate a series of events leading to rapid increase in intermediary metabolites (45). It is unlikely that TRIA itself, due to its lipophilicity, is moving rapidly in the plant. Rather this suggested the existence of a TRIA-mediated messenger that moves rapidly within the plant.

Chemical Messenger: TRIM

Root tissue of rice seedlings treated foliarly with the colloidal dispersion of TRIA was extracted to test for the presence of a messenger (48). A root extract of seedlings sprayed with the equivalent concentration of the dispersant used in the TRIA formulation was utilized as a control. Each extract was applied to the foliage of rice and corn seedlings. Dry weight increases were observed for both

species treated with the root extract of TRIA-treated plants but not with the control root extract. It was postulated that TRIA applied to the leaves elicited a message or chemical (TRIA-messenger: TRIM). TRIM moved from the shoots to the roots, and exogenous application of the extracted TRIM increased the growth of plants (48).

I. HIGHER PLANTS

INTRODUCTION

TRIA increases the growth and sometimes yield of several crop species. Several environmental factors and treatment conditions affect this response (5, 32, 39, 42, 47).

TRIM was shown to increase the dry weight of corn and rice seedlings in greenhouse bioassays (48). In order to observe the yield response of plants to TRIM, several vegetable crops and wheat were screened in the field. Interactions between treatment conditions and plant response to TRIM were investigated and compared to known interactions between TRIA and these conditions. Similar experiments were conducted in the greenhouse measuring dry weight responses of higher plants to TRIA and TRIM. A comparison of exogenously applied TRIA and TRIM may reveal similarities in the response of higher plants.

MATERIALS AND METHODS

TRIM Extraction

Foliage of 10-12 day old rice seedlings was sprayed with 1.0 µg/l TRIA formulated as a colloidal dispersion. Control seedlings were sprayed with 0.01 µg/l TAS (the amount of surfactant in the

formulation). After 100 minutes roots were cut off and extracted in 0.02M phosphate buffer (1 g roots/ 4 ml) containing 1.0 mM mercaptoethanol, pH 7.0 at 4°C. The extract was centrifuged for 30 min at 10,000 rpm, and the supernatant extracted 3 times with chloroform, retaining the aqueous fraction. Both the control plant root extract (TAS) and the TRIA-treated plant root extract (TRIM) were autoclaved at 130°C and 150 KPa for 20 min and then frozen.

Throughout the latter part of this research, the soxhlet method of extraction was utilized to extract TRIM. The procedure was more efficient and the activity of the extract was similar (49). A methanol extract obtained after 90-120 min of soxhlet extraction was taken to dryness by rotary evaporation and reconstituted in distilled water (1 g/4 ml).

Field Tests

Field studies were conducted during 2 growing seasons. The site for most tests was East Lansing, MI on a Spinks sandy loam. Wheat was grown in Saranac and E. Lansing, MI on Matherton and Conover loams, respectively. Radishes were grown on a Carlisle muck soil at Fowlerville, MI.

Common commercial planting, cultural, and pest control practices for these crops were utilized. Guard rows were included at the perimeter of plots. Treatments were applied as foliar sprays in approximately 163 l of water per hectare, using a backpack sprayer with a galvanized metal boom and CO_2 as a propellant. Crops were harvested at the proper stage of maturity to determine marketable yield.

Statistical Procedures

Experimental designs utilized were randomized complete blocks or split-plots having 5-6 blocks. Data were subjected to analysis of variance. Statistical significance was determined with Fisher's LSD or by determining F values for orthogonal and non-orthogonal comparisons.

RESULTS AND DISCUSSION

Field Studies

Several crops were screened for yield increase by TRIM application in the field for two growing seasons. In 1984, wheat, muskmelons, bell peppers, cucumbers, tomatoes, field corn, sweet corn, and radishes were tested. In 1985, experiments included only wheat, muskmelon, and bell pepper. In some tests, TRIA was used as a treatment in order to compare TRIM with TRIA, or to test various TRIA formulations. Results will be presented here by crop. Data is not shown for those experiments showing no significant differences.

Wheat

Several experiments were conducted with 'Augusta' soft white winter wheat in each of 2 growing seasons. TRIM and several formulations of TRIA were compared in 1984 at Saranac, MI. No differences in yield were found between an unsprayed control and foliar sprays of TRIM, TRIA colloidal dispersion prepared by the Proctor and Gamble Corp., TRIA colloidal dispersion prepared in our lab, TRIA formulated in the PRC as a coagulum, and a TRIA capsule formulated in the PRC.

In 1985, various concentrations of 3 different TRIA formulations were compared. Harvested seed size was increased by TRIA treatment (Table 1). The acetone-$CaCl_2$ formulation (55) appeared to be most effective for increasing yield. Yield was increased in a linear fashion with increasing TRIA concentration. Concentrations of 100 and 1000 µg/l of this formulation increased yield approximately 9% over the unsprayed control.

A comparison of an unsprayed control, TAS control and TRIM (each at 20 mg/l), and TRIA colloidal dispersion at 1 µg/l showed no differences in yield in 1984 at the 2 locations.

TRIM was tested when applied at various stages of plant development in 1984. Boot stage, anthesis, and post-anthesis applications of 20 mg/l TRIM were compared in all possible combinations. TRIM applications decreased yield compared to the control. These results are not surprising when considering the water stress imposed upon this wheat. Very little rain during the growing season limited water availability to such an extent that flag leaves rolled during midday heat and lower leaves senesced before grain-filling was complete. Biernbaum (5) found that stimulation by TRIA was limited under environmental conditions imposing a water stress. Regehr (39) observed increased yield of field corn by TRIA only when irrigation was used.

Yield and seed weight were measured in a dose response with TRIM applied once or twice during the 1985 growing season. Seed weight was increased over the control by 2 applications of TRIM but not by 1 application of TRIM; and seed weight increased linearly with increasing

Table 1. Response of 'Augusta' wheat to various concentrations of 3 different TRIA formulations

TRIA Formulation	µg/l	Yield (kg/ha) [a]	Wt./100 seeds (g) [b]
Untreated control	-	5139	4.45
Colloidal dispersion	0.1	5158	4.60
"	1.0	5360	4.65
"	10.0	4885	4.56
"	100.0	5014	4.57
Chinese coagulum	1.0	5209	4.55
"	10.0	5011	4.61
"	100.0	5393	4.59
"	1000.0	5126	4.60
Acetone-$CaCl_2$	10.0	4894	4.49
"	100.0	5564	4.62
"	1000.0	5634	4.61

a F value for linear trend with TRIA in acetone-$CaCl_2$ significant at 5% level.

b F value for control vs. TRIA treatments significant at 1% level.

TRIM concentrations (Table 2).

Muskmelons

Since plants were found to respond best to TRIA when applied during late afternoon, an experiment was designed to test the optimum time of day to spray TRIM. 'Summet' muskmelon plants were treated 2 times throughout the flowering period at 0900 hr, 1300 hr, 1700 hr, and 2100 hr in 1984. Yield, number of fruit, and average fruit size were determined. There were no differences in yield or number of fruit throughout the season, but larger fruit were produced when plants were sprayed with 20 mg/l TRIM at 0900 hr. Fruit size increased 11% in the early harvest and 8% in the total harvest (Table 3).

Muskmelons sprayed in 1985 with TRIA (1 µg/l) and TRIM (20 mg/l) once and twice during the season showed no differences in yield, fruit number, or average fruit size. These melon plants were severely stressed by a windstorm shortly after transplanting. Many plants sustained considerable damage to stems, leaves, and growing points, but eventually recovered. Plants were reassigned to blocks by size and treatments applied only after recovery, however, plants appeared to be quite variable in growth during the season. This variability could have masked differences due to treatment effects.

Bell Peppers

The response of 'Early Calwonder' bell peppers to TRIA and TRIM applied at different stages of crop development was studied in 1984. TRIA (1 µg/l) and TRIM (20 mg/l) were each applied during early flowering and fruit set. In the first 3 harvests, yield and number of fruit were decreased by any treatment with TRIA or TRIM. (Table 4). In

Table 2. Effect of increasing TRIM concentration on wheat seed weight

(mg/l) TRIM	No. of Applications	Yield (kg/ha)	Wt./100 seeds (g) [a]
0	-	4762	4.40
1	1	5049	4.42
10	1	5030	4.52
100	1	5080	4.47
1	2	4630	4.46
10	2	4986	4.52
100	2	4945	4.53

a F value for control vs. 2 applications of TRIM significant at 5% level

F value for linear trend with 2 TRIM applications significant at 5% level

r = 0.98** for the relationship between yield and seed weight

Table 3. Effect of TRIM (20 mg/l) applied at various times of day on fruit size of 'Summet' muskmelons

Time of TRIM application (hr)	Time of harvest			
	Early	Middle	Late	Total
		(kg/fruit)		
control	1.23	1.21	1.19	1.25
0900	1.37	1.32	1.41	1.35
1300	1.24	1.30	1.34	1.26
1700	1.22	1.35	1.24	1.25
2100	1.27	1.26	1.39	1.29

a, b F value for control vs. TRIM application at 0900 hr significant at 5% and 1% level, respectively.

Table 4. Response of 'Early Calwonder' bell peppers to application of TRIA and TRIM at 2 stages of development

Treatments		First 3 harvests			Final harvest			Total of all harvests		
Chemical	Stage of development	Yield (MT/ha)	Fruit # X 1000	Fruit size (g)	Yield (MT/ha)	Fruit # X 1000	Fruit size (g)	Yield (MT/ha)	Fruit # X 1000	Fruit size (g)
none	-	12.8[a]	113[a]	114	3.8	35	106	16.5	148	112
TRIA	flowering	11.3	97	117	5.6[a]	49[a]	115	16.9	145	116
"	fruiting	10.9	94	115	4.7	41	114	15.6	135[a]	116
TRIM	flowering	11.3	99	114	4.7	42[a]	112	16.0	141	113
"	fruiting	11.1	92	119	4.1	37	111	15.2	128	118[a]

a F value for control vs. treatment(s) significant at 5% level.

the last harvest of the season the yield was increased 47% by treatment with TRIA at flowering, and fruit number was increased by both TRIM and TRIA application at flowering. When all harvests were combined, it was observed that TRIM applied during fruiting decreased the number of fruit produced, but the size of these fruit was increased. The foliar applications were applied during late afternoon on warm sunny days. However, after the third harvest all TRIA and all TRIM treated plants were sprayed again with TRIA and TRIM, respectively, at 0900 hr. The treatment effects measured in the last harvest may have been a result of this final spray application, or may have resulted from the original treatments. An experiment was designed with peppers in 1985 to further study this effect. A split-plot design was utilized. The main plots were time of day for application and sub-plots were TRIM (20 mg/l) and TRIA (1 µg/l) applied during early flowering and early fruit set. Yield, number of fruit, and average fruit size were compared for an early harvest and a late harvest. There were no differences in yield or number of fruit for either harvest, but TRIM treatments at 1700 hr in the later harvest resulted in 7% larger fruit than the control (Table 5). Comparing the control with treatments of TRIA and TRIM during flowering at 1700 hr resulted in 9% larger fruit in the later harvest and 7% when combining both harvests. No differences resulted from the 0900 hr applications. Based on the 1985 data, the increases observed in the final harvest of the 1984 experiment were not a result of the late season spray being in the morning. These increases may have been a result of the late season spray occurring during a late stage of crop development, however, these increases may also have

Table 5. Response of 'Early Calwonder' bell peppers sprayed at 0900 hr and 1700 hr to TRIM and TRIA applied at 2 stages of development

Treatment			Fruit size		
Time of day sprayed	Chemical	Stage of development	Harvest 1	Harvest 2[a]	Total[b]
0900 hr	none	-	142	153	146
"	TRIA	flowering	136	150	146
"	"	fruiting	141	149	147
"	TRIM	flowering	149	153	148
"	"	fruiting	147	144	148
1700 hr	none	-	135	143	140
"	TRIA	flowering	138	156	153
"	"	fruiting	140	150	146
"	TRIM	flowering	135	158	147
"	"	fruiting	139	150	146
LSD 5%			ns	14	10

a F values for control vs. TRIM treatments at 1700 hr and control vs. flowering treatments at 1700 hr significant at 5% level.

b F value for control vs. flowering treatments at 1700 hr significant at 5% level.

occurred without the late season spray.

Cucumbers, Tomatoes, Field Corn, Radishes, and Sweet Corn

Multiple applications of TRIA have not stimulated growth beyond the extent that single applications have (43). However, multiple applications of TRIM do increase growth over a single TRIM application (48). In 1984, 'Castlehy 2012' pickling cucumbers were treated with a single application of TRIM, several applications of TRIM (20 mg/l), or TRIA colloidal dispersion (0.1 µg/l). There were no differences in yield or number of fruit between treatments for any fruit size.

'Ohio 7870' processing tomatoes were treated with TRIM applied once during vegetative growth, once at anthesis, 4 times during the season, and the TAS control applied 4 times during the season (20 mg/l). There were no differences in yield, number of fruit, or average fruit size.

'Great Lakes 5922' field corn was treated with TRIA (1 µg/l), TRIM (20 mg/l)applied once, twice, and the TAS control (20 mg/l) applied twice. There were no differences due to treatment in yield, number of ears, or average ear size.

In order to determine whether growth increases of roots would occur due to foliar applications of TRIM, 'Fancy Red' and 'White Icicle Short Top' radishes were treated with TRIA (1 µg/l), TRIM applied once, and TRIM applied twice (20 mg/l). No differences in average root fresh weight or average shoot dry weight were observed between treatments.

TRIM wastested when applied to 'Apache' sweet corn to determine the optimum stages of plant development for TRIM application. Early vegetative stage, anthesis, and ear-filling stage applications of TRIM

(20 mg/l) were compared in all possible combinations with an unsprayed control and a single early vegetative application of TRIA (1 µg/l). No differences were measured in yield, number of ears, or average ear size.

Greenhouse and Laboratory Bioassays

The majority of bioassays conducted in the greenhouse and growth chamber failed to exhibit significant increases in dry weight due to treatment with TRIA or TRIM. Considering 41 experiments with coefficients of variation (cv's) below 12%, only 6 resulted in significant increases due to TRIA or TRIM treatment. Cv's ranged from 3.0 to 11.6% and averaged 7.4%. Comparison of the interaction between various environmental factors and plant response to TRIM with the interactions between these factors and TRIA response was not feasible since experiments were not consistently reproducible.

CONCLUSIONS

Field tests indicated that there are some similarities and differences in the response of plants to TRIA and TRIM. Three of the 8 crops screened in the field for effects of TRIM responded favorably. Bell pepper experiments confirmed past research indicating that yield increases by TRIA are primarily a result of an increase in fruit number. Bell peppers tended to respond favorably to TRIM and to TRIA late in the season. There are several possible explanations for this late season response: (a) the effect of anthesis sprays may only be manifest on these yield parameters late in the season (delayed

maturity); (b) late season sprays of TRIM may increase the effectiveness of anthesis sprays; or (c) late season application, by itself causes the increase. Adequate soil moisture appears to be an environmental parameter favoring response to both TRIM and TRIA. Muskmelon fruit size was increased by TRIM application in the morning, but not in the afternoon. TRIA applications, however, have been recommended for afternoons (5, 40), and melon yield increases by TRIA have been a result of increased number of fruit rather than size (5). Single applications of TRIA have been as effective as multiple applications (43). However multiple TRIM applications were more effective than single applications in increasing wheat seed size. In these field studies both TRIA and TRIM did not consistently increase crop growth or yield. While in several experiments TRIM increased some yield parameters, these increases were not large enough to be commercially practical.

The lack of response by cucumbers, tomatoes, field and sweet corn, and radishes to TRIA or TRIM is not surprising considering TRIA's history of inconsistency, particularly in the field when measuring yield. Significant yield increases have been measured with these and many other crops by other researchers, however no crop species has responded consistently to TRIA. The lack of response may be due to environmental growth conditions, application technique, or environmental contaminants interacting with TRIA/TRIM. These explanations for TRIA and TRIM's inconsistency may also apply to greenhouse bioassays.

It proved impractical to use higher plants for comparing the

effects of TRIA and TRIM in this study. A simple, uniform system that would respond consistently to TRIA or TRIM was needed to carry out this research. A bioassay using Chlamydomonas reinhardtii was developed (56) which met these requirements. Results using this bioassay are presented here in section II.

II. CHLAMYDOMONAS EXPERIMENTS

INTRODUCTION

Synchronous cultures of algae are particularly useful for studying effects of biologically active chemicals such as TRIA at various stages of cell development. Algal cells may be synchronized by diurnal alternation of light and dark (53). These cells undergo the developmental and physiological changes leading to cell division at approximately the same time as they progress through their life cycle. The life cycle of unicellular algae has been studied extensively. In synchronous culture, cells assume 2 forms, referred to as dark cells and light cells (53). After cell division, near the end of the dark period, daughter cells are liberated. These "nascent dark cells", upon illumination become "active dark cells" (35). These are small, contain relatively large amounts of chlorophyll, and are most photosynthetically active (53). They increase in mass as they progress through the "growing phase" while illuminated (35). When photosynthetic activity decreases and the synthesis of nuclear material (ripening) begins, cells are referred to as "light" cells. Respiratory activity increases (36, 50), the nucleus becomes diffuse, and cells lose their motility (2). The duration of illumination after ripening is initiated determines the extent to which cells will divide (e.g. 2, 4, 8 daughter cells) (35). When cells enter the dark period, mitosis

begins and cleavage lines develop (2). Nuclear division, chloroplast division, and cytokinesis take place, but in a less synchronous manner than the ensuing cell division (34). Distinct cell outlines appear, and daughter cells increase in size until the mother cell wall ruptures and new nascent dark cells are released (2). Division of light cells into dark cells can proceed only under aerobic conditions (16).

If cells cultured in a light-dark cycle are placed under conditions of continuous illumination, (as was the case with these experiments) cells will divide synchronously in the light as they would if in the dark for the first cycle of continuous illumination. Synchrony is not lost until the second cell cycle in continuous light (2, 9, 34).

MATERIALS AND METHODS

Chlamydomonas Bioassay

Synchronous cultures of Chlamydomonas reinhardtii Dangeard, (-) strain (N. 90), a unicellular green alga, were grown in 2.8 liter Fernbach flasks containing 2 l of nutrient solution (Table 6), continually aerated. Cultures received 100-300 $\mu Es^{-1}m^{-2}$ light from fluorescent bulbs during an 8 or 12 hr photoperiod. Temperature was maintained at 30°C. Cultures were renewed with fresh nutrient solution every 7 days. No cultures older than 5 days were used for bioassays. Bioassays were initiated 30-120 min after the initiation of the light cycle. Cells were diluted in enough nutrient solution to obtain an O.D. of 0.05 to 0.06 at 652 nm on a spectrophotometer (4.0×10^5 to 6.0×10^5 cells/ml). Before the initiation of the experiment, 100 ml CO_2/l

Table 6. Nutrient solution for Chlamydomonas reinhardtii [a]

Stock solutions	Composition of stock solution (g/l)	Amount of stock for nutrient solution (ml/l)
KPi Solution		10.0
K_2HPO_4	14.38	
KH_2PO_4	7.26	
Beijerinck's Solution		50.0
NH_4Cl	8.00	
$CaCl_2 \cdot 2H_2O$	1.00	
$MgSO_4 \cdot 7H_2O$	2.00	
Tris Cl Solution		10.0
Tris basic (to pH 7.5 with HCl)	242.00	
Hunter's Trace Elements		1.0
EDTA (Ethylenediamine tetracetic acid)	50.00	
$ZnSO_4$	22.00	
H_3BO_4	11.40	
$MnCl_2 \cdot 4H_2O$	5.10	
$FeSO_4 \cdot 7H_2O$	5.00	
$CaCl_2 \cdot 6H_2O$	1.60	
$CuSO_4 \cdot 5H_2O$	1.60	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (to pH 6.5 - 6.8 with KOH)	1.10	

a (56)

was bubbled through the cell suspension for 30 seconds. Ten ml aliquots of the suspension were pipetted into disposable culture tubes (16 X 100 mm) containing the extract or chemical to be tested. Cells were cultured in these tubes under continuous light (150 $\mu Es^{-1}m^{-2}$) at 30°C. After 16-24 hr, tubes were centrifuged at 3000 rpm for 15 min and the algae pellet resuspended in 5 ml of 80% acetone. Tubes were again centrifuged at 3000 rpm for 5 min. Chlorophyll readings were taken at 652 nm, and zero time O.D. was subtracted from these readings to estimate increase in growth. Chlorophyll was determined by Arnon's (1) method.

In tests where cell density was determined, representative samples of the cell suspension were removed from bioassay tubes prior to centrifugation. Cells from the sample were counted using a hemacytometer, and the number of cells per ml calculated. Kodak tungsten film, ASA 160 was used for photographs of cells magnified 1000X with a Nikon light microscope in the photographic examination.

Preparation of TRIA and TRIM for Bioassay

The TRIM used in this bioassay was prepared as previously described (page 12).

The TRIA used for most comparisons was not formulated by any method previously described. The colloidal dispersion, which was effective at concentrations of 1 mg/l in another Chlamydomonas bioassay utilizing a continuous supply of CO_2 (21), was not effective in this bioassay at concentrations low enough to be practical. Excessive quantities of the 1000 mg/l dispersion are required to achieve concentrations of 100 mg/l, so a procedure using pure TRIA was

developed for this Chlamydomonas bioassay (38). TRIA crystals added to culture tubes float to the top after the cell suspension is added. During incubation, cells settle to the bottom of the tube where TRIA is unavailable. The procedure employed in this study allows the TRIA to remain in the bottom of the culture tube where it comes into contact with cells. TRIA crystals were dissolved in warm chloroform and the appropriate quantity of solution added to each culture tube. This solution was allowed to evaporate by placing tubes in a forced air oven at 70°C until all traces of chloroform evaporated. This resulted in aggregates of TRIA adhering to the bottom of culture tubes.

Statistical Procedures

Experimental designs utilized were randomized complete blocks or split-plots having 3-4 blocks. Data were subjected to analysis of variance and means compared using Fisher's LSD or with orthogonal and non-orthogonal tests.

RESULTS AND DISCUSSION

Comparisons of Stimulation by TRIA and TRIM

Throughout this study, TRIA treatment of Chlamydomonas cultures resulted in chlorophyll increases ranging from 3 to 65%, averaging 18%. TRIM treatment increased chlorophyll 20 to 142%, and averaged 64%. These increases were measured against a water control within 16-24 hr of treatment. Several factors affect this increase, including concentration, formulation, presence of CO_2, time of incubation, temperature and light intensity, and the growth stage that the cells

are treated.

Unfortunately, this Chlamydomonas bioassay is not sensitive to differences between the TAS control crude extract and the TRIM crude extract. Fractions of crude TRIM, separated by HPLC, have shown increases in chlorophyll over the equivalent fractions of the control (37), but since availability of these fractions is limited and identity unknown during the course of this research, only the crude TRIM extract was used for comparison with TRIA. TAS extracts do not increase the growth of whole plants such as rice and corn, which do respond to TRIM (48).

A dose response of Chlamydomonas with the colloidal dispersion of TRIA shows chlorophyll increases at 100 mg/l (Table 7).

A dose response of Chlamydomonas with TRIA aggregates, as described shows increases in chlorophyll and cell density at 1-100 mg/l (Table 8). There is a linear trend for both cell density and chlorophyll. The chlorophyll content was related to cell density ($r = 0.98^{**}$).

The TRIA crystals at 1, 10, and 100 mg/l increased growth over the colloidal dispersion by 13, 24, and 20%, respectively (Figure 1).

As the concentration of TRIM increases, the chlorophyll and cell density of Chlamydomonas cultures increases (Table 9). This is consistent with findings of researchers in the process of purifying and identifying TRIM (37).

Table 7. Response of Chlamydomonas cultures to the colloidal dispersion of TRIA (average of 2 experiments)

TRIA (mg/l)	µg chlorophyll/ml Chlamydomonas[a]
0	0.826
0.001	0.841
0.01	0.841
0.1	0.870
1.0	0.855
10.0	0.826
100.0	0.928**
LSD 5%	0.058
LSD 1%	0.087

a F value for linear trend of increasing chlorophyll with increasing concentration significant at 1% level.

Table 8. Response of Chlamydomonas cultures to pure TRIA (average of 2 experiments)

TRIA (mg/l)	µg chlorophyll/ml Chlamydomonas	Cells/ml X 10^5
0	0.899	9.3
0.001	0.913	11.0
0.01	0.899	9.7
0.1	0.942	10.4
1.0	1.014	16.4
10.0	1.058	17.9
100.0	1.130	20.8
LSD 5%	0.087	4.2
LSD 1%	0.116	5.6

$r = 0.98^{**}$ for relationship between chlorophyll and cell density

F value for linear trend of increasing chlorophyll and cell density with increasing concentration significant at the 1% level

Figure 1. The response of Chlamydomonas to the colloidal dispersion of TRIA and TRIA crystals at various concentrations. F value for the interaction between TRIA formulation and concentration is significant at the 1% level. LSD between concentrations at 5% is 0.058 and at 1% is 0.087. (Average of 2 experiments)

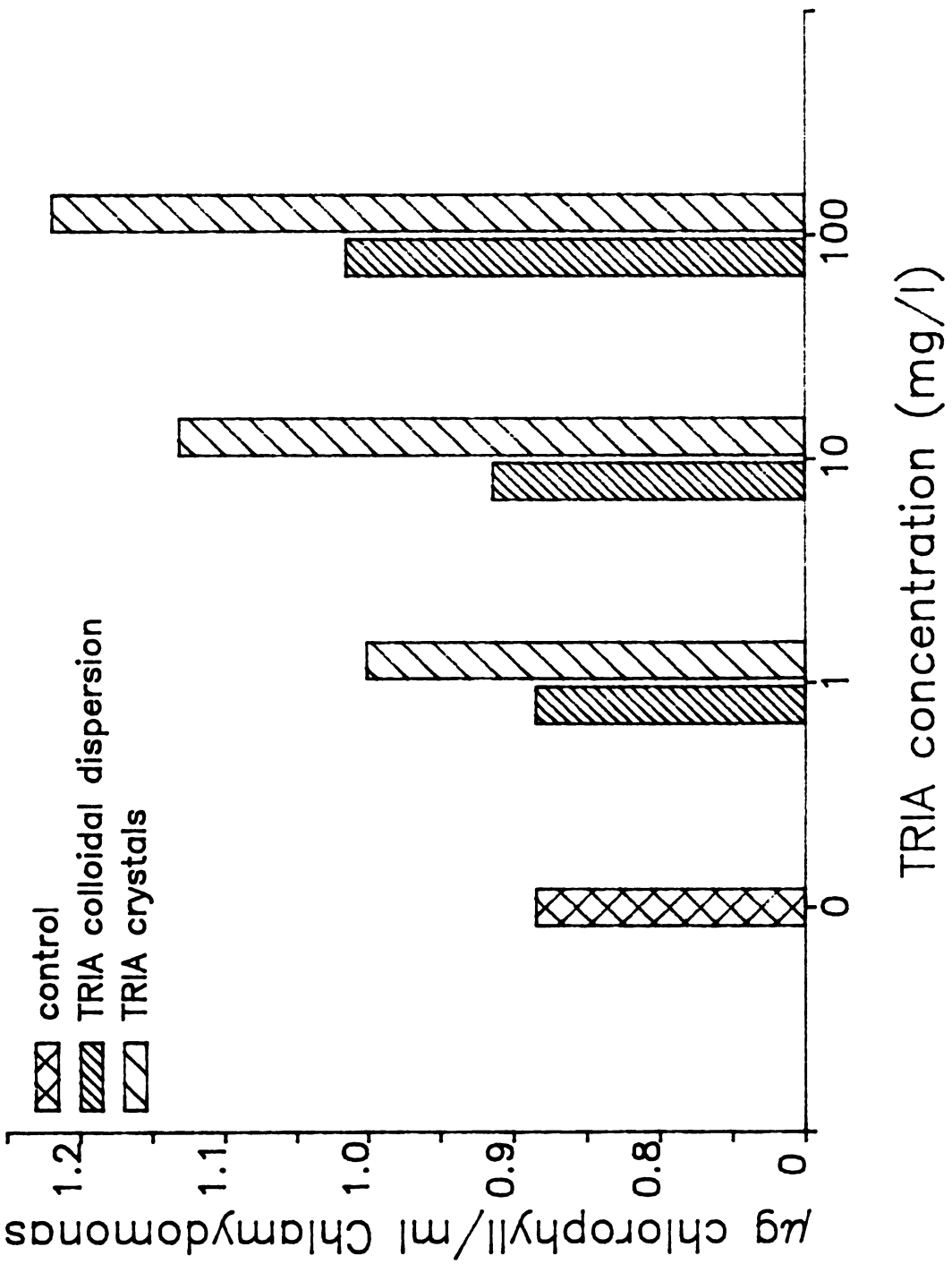

µg chlorophyll/ml Chlamydomonas
1.2
1.1
1.0
0.9
0.8
0
control
TRIA colloidal dispersion
TRIA crystals
0
1
10
100
TRIA concentration (mg/l)

Table 9. Response of Chlamydomonas cultures after incubation for 16-20 hr with various concentrations of TRIM

TRIM (mg/l)	µg chlorophyll/ml Chlamydomonas [a]	Cells/ml X 10^5
0	0.725	23.6
2	0.768	29.1
20	0.855	28.2
200	1.449	31.9
LSD 5%	0.174	-
LSD 1%	0.232	-

a F value for linear trend of increasing chlorophyll with increasing concentration significant at 1% level (average of 4 experiments

b F value for control vs. TRIM treatments significant at 5% level.

F value for linear trend of increasing cell density with increasing concentration significant at 5% level (average of 2 experiments)

Interaction of CO_2 with TRIA and TRIM

Increases in photosynthetic CO_2 assimilation of Chlamydomonas cells incubated with TRIA were observed only when cells were cultured with CO_2 (21). In the Chlamydomonas bioassay employed in this study, increases in chlorophyll were observed in cells incubated without CO_2, but only when CO_2 was bubbled through the cell suspension shortly before incubation with TRIA. TRIA treatment resulted in increases in chlorophyll of 16 to 50% over a control when 100 ml CO_2/l was bubbled through the cell suspension for 30 sec (Table 10). In most cases, the TRIM response occurred without addition of CO_2 to the media, but chlorophyll increase over zero time was poor. The TRIA response occurred only if the interval between addition of CO_2 to the cell suspension and initiation of TRIA incubation was not longer than 1 hr. Chlorophyll increases by TRIM treatment declined steadily as the time interval after purging with CO_2 increased, but remained higher than the control (Table 11).

In this bioassay, cells are cultured without supplemental CO_2 up until the time of treatment. Air-grown cells are reported to have an increased affinity for CO_2 since they have adapted to conditions where CO_2 is limiting (3). The Chlamydomonas cells used in this study must utilize the burst of CO_2 they receive very efficiently. The CO_2 added to t the cell suspension is likely to be consumed and some to dissipate from culture tubes within 2-3 hr. The failure of TRIA to elicit a response in cultures receiving no added CO_2 or in cultures where CO_2 is no longer available suggests that CO_2 is required for the TRIA response. This is in agreement with other researchers, reporting a

Table 10. Effect of TRIA on Chlamydomonas cultures with and without CO_2 purged into the media

TRIA (mg/l)	CO_2	µg chlorophyll/ml Chlamydomonas	% increase over zero time
0	-	0.072	7.7
1	-	0.058	7.3
10	-	0.058	6.9
100	-	0.058	7.1
0	+	0.725	84.0
1	+	0.841	96.0
10	+	0.956	110.2
100	+	1.087	125.4
LSD 5%		0.044	4.8
LSD 1%		0.058	6.4

a F value for the interaction between CO_2 and TRIA treatment significant at 1% level.

Table 11. Response of Chlamydomonas cultures to treatment with TRIA and TRIM various times after purging with CO_2

Time after purging with CO_2 (min)	µg chlorophyll/ml Chlamydomonas		
	control	TRIA (100 mg/l)	TRIM (20 mg/l)
2	1.014	1.174	2.246
6	1.029	1.130	2.116
18	1.014	1.188	2.072
54	0.971	1.116	1.884
162	0.797	0.783	1.522

LSD between times, 5% = 0.116
1% = 0.145

LSD between treatments, 5% = 0.101
1% = 0.145

Interaction between time after CO_2 addition and treatment significant at 1% level.

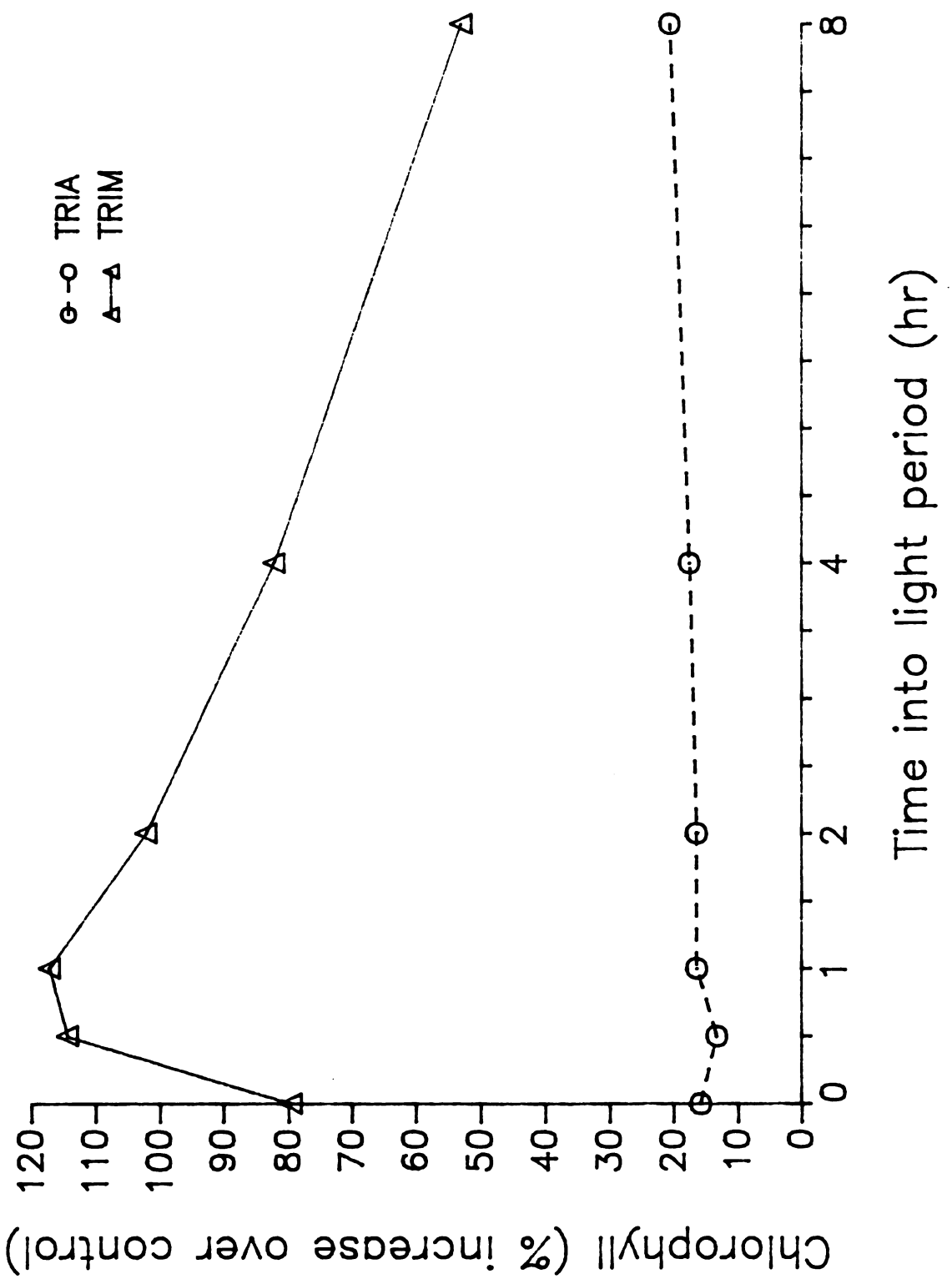
possible regulatory role of CO_2 in the mode of action of TRIA (6, 21, 45). Chlamydomonas cells grown in high CO_2 have excess carbon reserves, whereas air-grown cells do not. TRIA may enhance the ability of high CO_2-grown cells to mobilize these storage reserves (21).

Optimum Stage of Cell Cycle for Treatment

Cultures were treated with TRIA and TRIM (100 mg/l) at various times after initiation of the light period to test whether differences in response occur as a result of treatment at different stages of cell development. The greatest increases by TRIM were observed using cultures treated 0.5, 1.0, and 2.0 hr into the cell cycle (Figure 2). After 1 hr, percent increase gradually declined as cells advanced through the cycle, but treated cultures were higher in chlorophyll concentration than controls. Cells treated at the initiation of the light period exhibited a relatively low response. This pattern indicates that active dark cells respond better to TRIM than nascent dark cells (0 hr) or light cells (4 and 8 hr). The greatest response occurred when cells are most photosynthetically active and when respiration rate is low (36). There was relatively little change in response to TRIA over time, but a slight increase is observed from 0.5 to 8 hr, as the cells' starch reserves build up.

The decline in response to TRIM as treatment time after addition of CO_2 to the culture increased (Table 11) may be related to the stage of the cell cycle at treatment. However, since a TRIA response is evident here (Figure 2) even with treatment 8 hr into the cell cycle, the absence of response to TRIA in the CO_2 experiment (Table 11) was not a result of the cells' developmental stage, but rather the

Figure 2. Effect of TRIA and TRIM (100 mg/l) when applied at various stages of the cell cycle. F value for chlorophyll content between control and each treatment at each time significant at the 1% level. F value for interaction between time into the light period and treatment significant at the 1% level. (TRIA percentages represent average of 2 experiments; TRIM percentages represent average of 3 experiments)


TRIA
TRIM
Chlorophyll (% increase over control)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
4
8
Time into light period (hr)

depletion of CO_2 from the cell suspension.

Stage of Cell Cycle When Response is Observed

Chlamydomonas cells were counted and chlorophyll determined after various periods of incubation with TRIA and TRIM in order to determine when increases took place. Incubation was initiated at the beginning of the light cycle, when cells of the synchronous culture are becoming photosynthetically active. The largest chlorophyll increase due to TRIA or TRIM treatment took place after 8 hr of incubation and leveled off after 16 hr (Figure 3a). Cell density increased after cells were given time to divide (16 hr after initiation of the light cycle) (Figure 3b). In this culture system, synchronous cell division occurs at 16 hr (discussed under "Photographic Examination"). In this test, cell density was determined several hours after termination of incubation. A sample of cells to be counted was removed from each culture tube and kept in a refrigerator (5°C) in the dark until a more convenient time. Preliminary tests have shown that no increases in chlorophyll take place for several hours under these conditions. However, all divided cells were released from the mother cells during refrigerated incubation. Chlorophyll was determined at exactly 16 hr. These results indicate that more daughter cells are produced from treated than from untreated mother cells (higher division number).

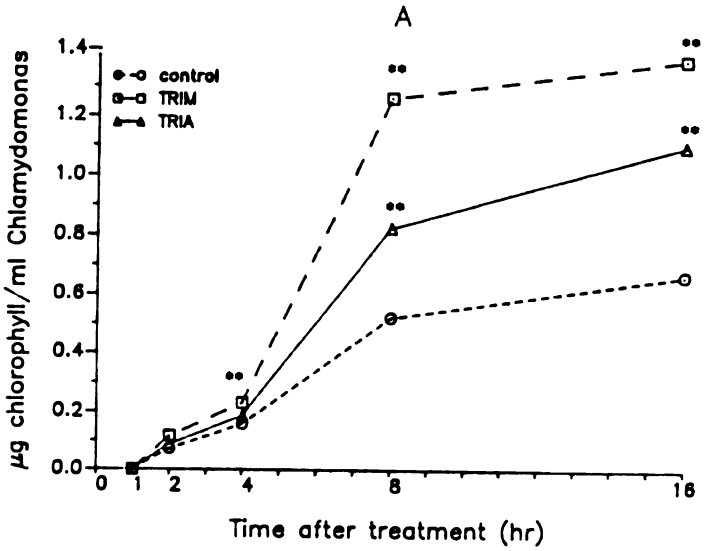
Chlorophyll per cell (calculated based on total chlorophyll in the system at the times indicated) increased in treated cultures between 4 and 8 hr during incubation, at which time the light-dependent processes of photosynthesis and formation of nuclear substances take place ("growth" and "ripening" phases, respectively, 35). The chlorophyll

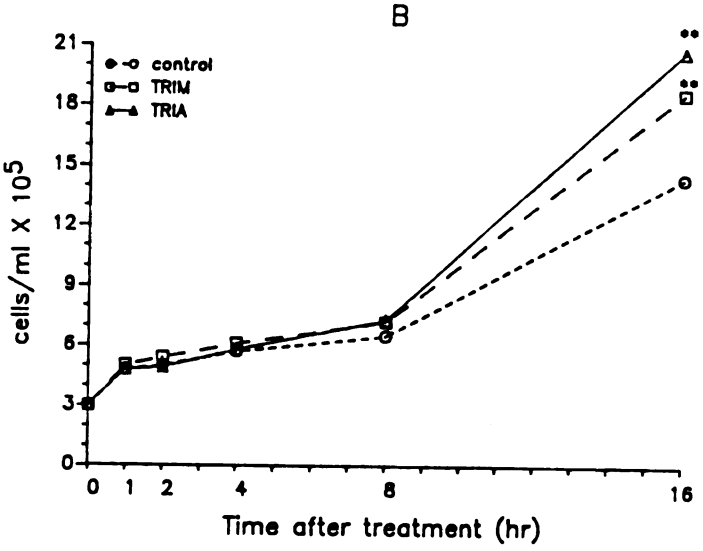
Figure 3. Effect of TRIA and TRIM (100 mg/l) on Chlamydomonas cultures at various times during incubation. ** signifies F value for difference from control significant at 1% level. (Average of 2 experiments)

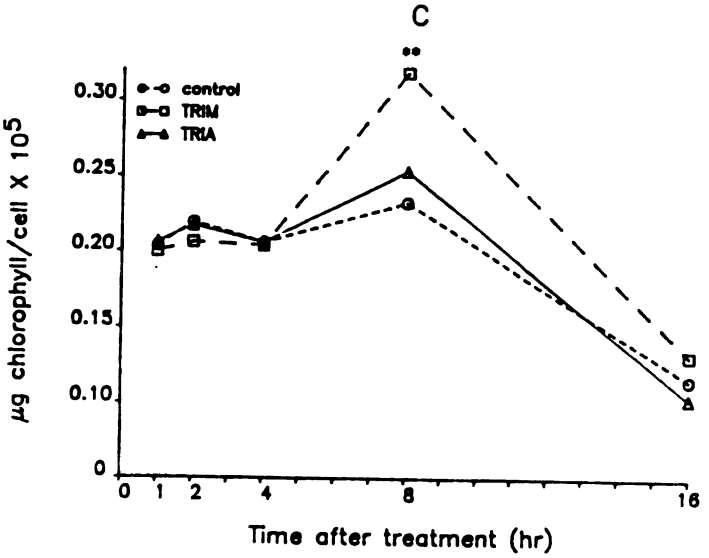
A. Increase in chlorophyll of Chlamydomonas cultures. LSD between treatments at a point in time is 0.058 at the 5% level and 0.072 at the 1% level.

B. Increase in cell density of Chlamydomonas cultures. LSD between treatments at a point in time is 1.3 at the 5% level and 1.8 at the 1% level.

C. Chlorophyll content of Chlamydomonas cells. LSD between chlorophyll contents of each treatment at different points in time is 0.032 at the 5% level and 0.043 at the 1% level.


A
control
TRIM
TRIA
µg chlorophyll/ml Chlamydomonas
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 4 8 16
Time after treatment (hr)


B
control
TRIM
TRIA
cells/ml X 10^5
21
18
15
12
9
6
3
0
0 1 2 4 8 16
Time after treatment (hr)


C
control
TRIM
TRIA
µg chlorophyll/cell X 10^5
0.30
0.25
0.20
0.15
0.10
0
0 1 2 4 8 16
Time after treatment (hr)

increase in TRIA and TRIM treated cells after 8 hr of incubation was greater than for treated cells incubated only 1, 2, or 4 hr. Chlorophyll per cell in controls was not significantly increased after 8 hr incubation over controls incubated 1, 2, or 4 hr (Figure 3c).

TRIM-treated cultures exhibited higher chlorophyll per cell after 8 hr than the controls, but chlorophyll per cell for TRIA-treated cultures was similar to controls.

It appears that TRIM affects chlorophyll content of cells, but that TRIA affects primarily cell division.

Environmental Effects

Light intensity and temperature during incubation affect response by Chlamydomonas to TRIA and TRIM. Light intensity is generally responsible for the amount of biomass produced in unicellular algae. Higher light intensities result in increased photosynthetic rate and growth, a greater cell volume prior to division, and greater division number (34, 35). This increase in cell size with increasing light intensity is accompanied by a decrease in chlorophyll concentration (33). This decrease in chlorophyll has been attributed to photoxidation of the pigment in cells grown at high light intensity (24). The rate of photosynthesis per cell or rate per unit chlorohyll, however, increases continually with increasing light intensity (33).

The effect of TRIA and TRIM on Chlamydomonas cells was studied at 4 different light intensities. The treated cultures remained higher in total chlorophyll than controls at all light intensities. Light did not affect the controls, however, both total chlorophyll and chlorophyll per cell in TRIA and TRIM treated cultures declined

relative to their controls with increasing light intensity (Table 12). TRIA increased cell density at all light intensities; TRIM increased cell density only at 450 $\mu Es^{-1}m^{-2}$. TRIM increased chlorophyll per cell at the lower light intensities, TRIA had no effet on chlorophyll content of cells. The diminished response to TRIA and TRIM at high light intensities may be a result of less chlorophyll synthesis or chlorophyll destruction at these light intensities.

Temperature affected chlorophyll production by TRIA and TRIM treated cultures. After 24 hr at 15°C, no increases in chlorophyll for controls or treatments were observed. As temperature increased, chlorophyll production increased for all treatments leveling off between 30 and 35°C (Figure 4). The largest increase over the control by TRIM was 101% at 30°C, and by TRIA, 27% at 25°C.

Researchers studying the life cycle of Chlorella ellipsoidea (another green, unicellular alga) and Chlamydomonas reinhardtii in synchronous cultures determined that temperature affects the rate at which the cell cycle of synchronous cultures proceeds. Higher temperatures resulted in more rapid growth and ripening, smaller cell volume at initiation of cell division, and earlier onset of cell division. Temperature had no effect on division number (34, 35).

TRIM increased chlorophyll production to the greatest extent at temperatures where the most rapid growth occurs (30 and 35°C). At these temperatures, cells are likely to be smaller and contain less stored carbon than at 20 or 25°C. The greatest increase in chlorophyll due to TRIA occurred at 25°C, a temperature where cells would attain a greater volume and would have accumulated greater amounts of stored

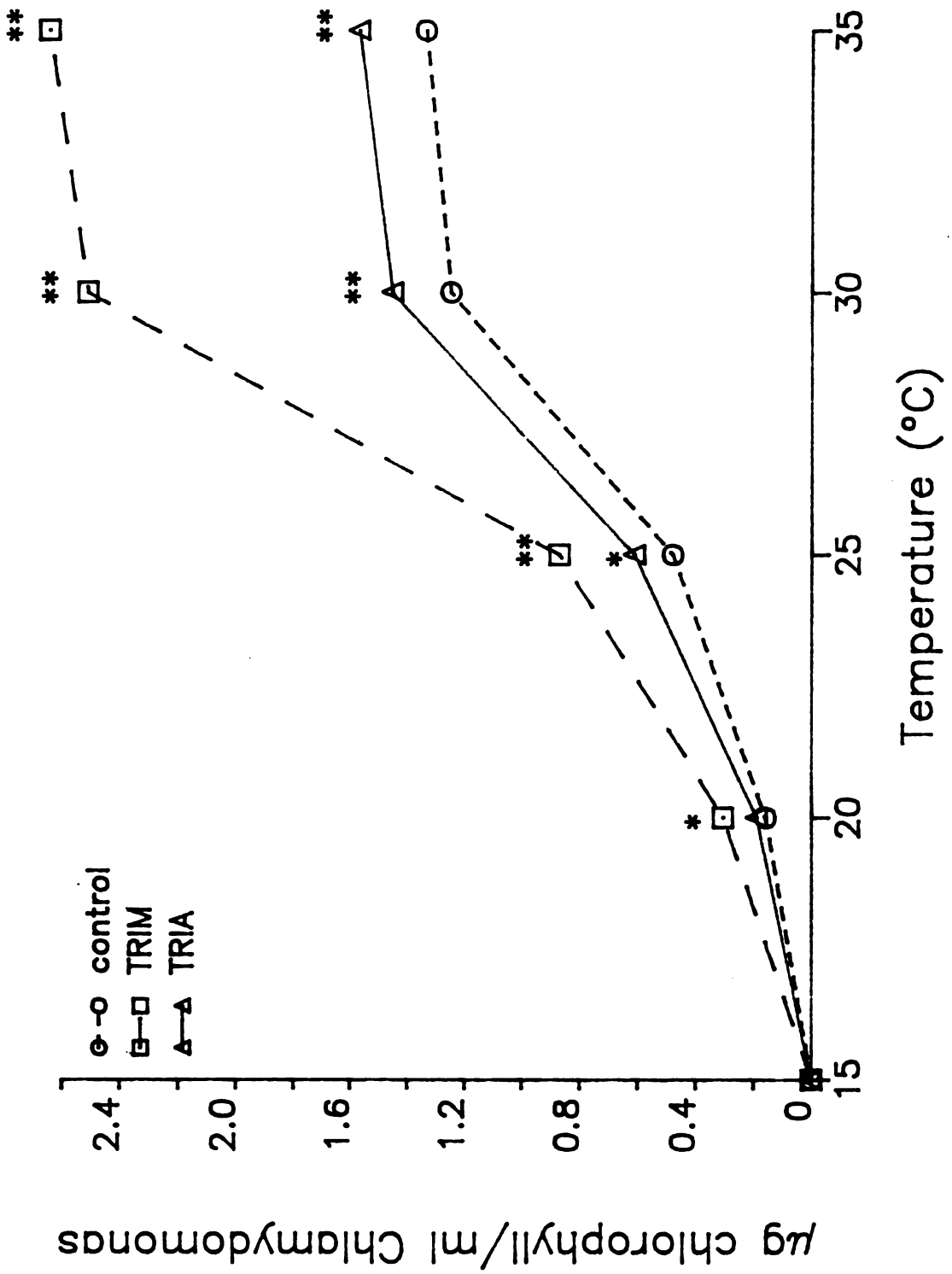
Table 12. Response of Chlamydomonas cultures to TRIA and TRIM (100 mg/l) after 20 hr at different light levels

Light Level ($\mu Es^{-1}m^{-2}$)	Treatment	µg chlorophyll per ml	Cells per ml X 10^5	chlorophyll per cell X 10^5
150	control	0.739	11.2	0.052
"	TRIM	1.362	16.1	0.069
"	TRIA	1.101	21.8	0.052
300	control	0.754	15.4	0.040
"	TRIM	1.087	18.9	0.054
"	TRIA	1.058	23.9	0.041
450	control	0.754	16.0	0.037
"	TRIM	1.072	23.0	0.042
"	TRIA	1.087	26.0	0.042
600	control	0.667	15.9	0.036
"	TRIM	0.898	21.0	0.036
"	TRIA	0.928	22.6	0.038
LSD 5%		0.130	5.2	0.014
LSD 1%		0.170	7.0	0.019

means of 2 experiments

data represent increases occurring after initiation of incubation

Figure 4. Response of Chlamydomonas cultures to TRIA and TRIM (100 mg/l) at different temperatures. F value for interaction between temperature and treatment significant at the 1% level. LSD between treatments at each temperature is 0.13 at the 5% level and 0.17 at the 1% level. LSD between temperatures for each treatment is 0.14 at the 5% level and 0.20 at the 1% level.


μg chlorophyll/ml Chlamydomonas
2.4
2.0
1.6
1.2
0.8
0.4
0
15
20
25
30
35
Temperature (°C)
control
TRIM
TRIA
*
**

carbohydrate.

Bittenbender, et al. (6) suggested that stored carbon was the substrate in the TRIA response, rather than the immediate products of photosynthesis. Carbohydrates produced by photosynthesis are stored in the cell, and are mobilized during nuclear division to provide energy for nucleic acid synthesis, deposition of cell wall, and nuclear division (50). If TRIA were affecting a stored product, a greater effect may be expected if more stored product is allowed to accumulate. This temperature data suggests that temperatures 20°C and below result in growth too slow for much carbohydrate accumulation, hence, small effect of TRIA. At temperatures 30°C and higher, growth is so rapid that cell division occurs before maximum photosynthate accumulation has been accomplished, decreasing the impact of TRIA.

The increase by TRIM in a situation of rapid growth when less stored carbon is present indicates that TRIM probably doesn't affect the stored carbon as TRIA does. TRIM may be directly affecting photosynthesis, increasing a cell's efficiency for storing carbon at high temperatures.

Effect of TRIA and TRIM Together

Combinations of TRIA and TRIM enhanced chlorophyll production in Chlamydomonas cultures. Incubation of cultures with both TRIA and TRIM resulted in greater chlorophyll production than an equivalent total concentration of TRIA or TRIM alone, but not greater than the sum of the two (Table 13). These results are difficult to explain without knowledge of the mechanisms by which TRIA and TRIM increase growth. It is possible that TRIM treatment ultimately results in increased stored

Table 13. Effect of TRIA, TRIM, and their cominations on chlorophyll content of Chlamydomonas cultures

Treatment	(mg/l)	µg chlorophyll/ml Chlamydomonas	% increase over control
control	-	0.855	-
TRIA	5	1.188	39
"	10	1.217	42
"	15	1.217	42
"	20	1.203	41
TRIM	5	0.986	15
"	10	1.043	22
"	15	1.116	30
"	20	1.217	42
TRIA + TRIM	2.5 each	1.159	36
"	5.0 each	1.275	49
"	7.5 each	1.333	56
"	10.0 each	1.391	63
LSD 5%		0.174	-
LSD 1%		0.203	-

carbon, which may be the substrate affected by TRIA. In contrast, TRIA treatment may cause rapid increases in the cellular biochemical components upon which TRIM acts. The rapid effect of TRIA is well documented. Increased photosynthesis in Chlamydomonas was observed within 1 hr of TRIA treatment (20). If TRIM is affecting photosynthetic rate, the effect of TRIM may be enhanced by TRIA's effect on stored carbon. And if the quantity of stored carbon produced is increased by TRIM, TRIA's effect will be compounded by the effect of TRIM.

Photographic Examination of Cells Throughout the Cell Cycle

Chlamydomonas cells were photographed at various intervals throughout a 24 hr period to observe changes throughout the cell cycle. Cultures incubated with 100 mg/l TRIA or TRIM were compared to a control culture. Incubation in culture tubes as in the Chlamydomonas bioassay was initiated 2 hr after the beginning of the light cycle. Photographs of cells in each culture were taken 0, 2, 5, 8, 12, 16, and 20 hr into the cycle. Several photographs are presented in Figure 5. At both 0 and 2 hr, cells were small and motile. From 5-8 hr, cells were considerably larger and in some, cellular material appeared quite diffuse. Cleavage lines were visible 12 hr into the cycle. By the 16th hr, cell division had begun. Distinct daughter cells were visible in the mother cells. Number of daughter cells ranged from 2-8. Several motile single cells, which had already completed the cycle were also observed. Cell division had been completed by the 20th hr, when only small, single, motile cells were present. No striking differences were observed due to treatment with TRIA or TRIM.

Figure 5. Various stages of the cell cycle of Chlamydomonas reinhardtii.

A. 2 hr after initiation of the light period, cells were small and motile.

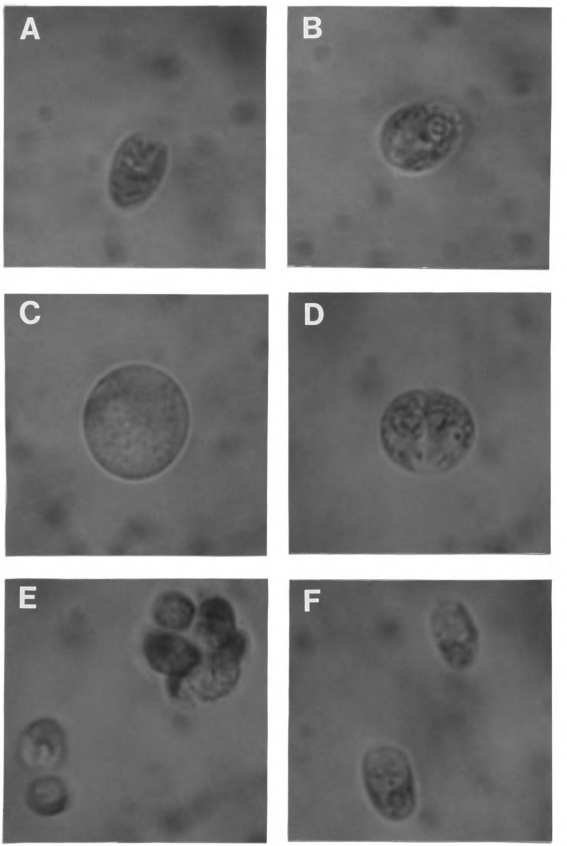
B. 5 hr after initiation of the light period, cells were larger.

C. 8 hr after initiation of the light period, cells appeared to have lost their motility.

D. 12 hr after the photoperiod was initiated, cleavage lines were observed in cells.

E. 16 hr after initiation of the light period, many cell clusters and several small single cells were present.

F. 20 hr after initiation of the light period, only newly divided "nascent dark cells" were observed.


A
B
C
D
E
F

There was considerable variability in synchrony, which made uniform observation difficult and may have masked observable differences due to treatment. Surzycki (52), studying synchronously grown cultures of Chlamydomonas reinhardtii found that variation in temperature of more than 1°C throughout the culture vessel resulted in poor synchrony. Water-jacketed culture bottles were utilized in his study to maintain uniform temperature. It is possible that some variation in temperature occurred in the 2.8 l flasks used to culture cells for bioassay in this study.

Surzycki maintained cultures at 21°C and found cell division to occur between 16 and 19 hr into the cycle. Cultures in this study, maintained at 30°C began cell division between 12 and 16 hr and completed division between 16 and 20 hr. This discrepancy in time of division is probably due to the difference in temperature in the 2 studies. Higher temperatures cause the course of the cell cycle to proceed more rapidly (34, 35).

CONCLUSIONS

Incubation of Chlamydomonas reinhardtii cells with both TRIA and TRIM resulted in increased total chlorophyll and cell density. Considering that different conditions of the cell cycle and environment facilitated optimum response for TRIM than for TRIA, the mode of action of exogenously applied TRIM may differ from that of TRIA. The algae consistently responded more to TRIM than TRIA based on chlorophyll measurements. The chemical structure(s) of TRIM may explain this difference in response between TRIA and TRIM. Other chemicals not

elicited by TRIA may also act directly or interact with TRIM to bring about this difference between TRIM and TRIA. The site of action for TRIA was proposed to be the plasma membrane (40). TRIA may trigger a cascade of events eventually altering the physiology and biochemistry of the cell, possibly affecting carbon storage. TRIM, unlike lipophyllic TRIA, is more likely to enter the cell. It may act directly on the photosynthetic mechanism.

Increasing the concentration of TRIM increased the response by the cells. Stimulation of growth at very low concentrations is characteristic of TRIA, but in this study relatively high doses were required to elicit a response. Houtz (21) reported decreased binding affinity of TRIA particles to cells cultured with low CO_2. The absence of continuous supplemental CO_2 in this bioassay may also have decreased the effectiveness of TRIA.

A single enrichment of Chlamydomonas cell suspensions with CO_2 enhanced the activity of TRIM, however TRIA did not stimulate growth without this CO_2 enrichment. This research with TRIA is in agreement with past research (20) suggesting that CO_2 is necessary for the TRIA response. Carbon dioxide is believed to play a regulatory role in the TRIA mode of action (6, 21).

TRIA is somewhat more effective when applied to Chlamydomonas cells late in the life cycle. However, the best response to TRIM by Chlamydomonas is observed early in the life cycle, when cells are most photosynthetically active. This supports the hypothesis that stored carbon is the substrate in the TRIA response, and it suggests that TRIM is affecting photosynthesis. TRIM may be increasing photosynthetic

activity, thus would be most effective if applied at the most photosynthetically active stage of the life cycle. TRIA may play a role in the mobilization of stored carbohydrates during nuclear division, thus producing a larger effect if applied at the stage of development when these carbohydrate reserves are being utilized.

Incubation with both TRIA and TRIM resulted in increased chlorophyll prior to synchronous cell division and greater cell density following this division. However, cell density increases by TRIA were of a greater magnitude that those of TRIM, and chlorophyll increases by TRIM were greater than those by TRIA. These experiments suggest that TRIA affects primarily cell division by increasing the number of divisions, whereas TRIM affects chlorophyll production per cell.

Manipulation of environmental parameters affects the way cells responded to TRIM and TRIA because the environment alters the cell cycle. Higher light intensity increases photosynthesis and higher temperatures shorten the time to cell division. Conditions that are optimum for the cellular processes affected by TRIA and TRIM probably facilitate the greatest response by the cell to these growth stimulators.

TRIA and TRIM do not appear to synergistically stimulate the growth of Chlamydomonas cells, but rather the effect of one is enhanced by the effect of the other. It is possible that in these cultures, both cell density and chlorophyll per cell were increased.

TRIA may initiate the synthesis of a chemical messenger(s) which may be responsible for many of the responses previously credited to TRIA. In these experiments, the response of algae to TRIM differed

from that of TRIA. Whether TRIM is entirely responsible for the effects observed following exogenous TRIA application is inconclusive. An understanding of the physiological pathway by which TRIM is elicited may reveal exactly what events take place in plants in response to TRIA.

The bioassay utilized in this study is a useful tool for studying the effects of TRIA on living plant cells for several reasons: (a) the biochemistry of photosynthesis in this alga is similar to that in higher plants, (b) the response to TRIA is consistent and easily measured, (c) cultures are easily maintained, (d) measurable differences in growth occur within 24 hr, and (e) large numbers of treatments can easily be handled.

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