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DEVELOPMENT AND CHARACTERIZATION OF AN IN VITRO SYSTEM RESPONSIVE TO 1-TRIACONTANOL

presented by

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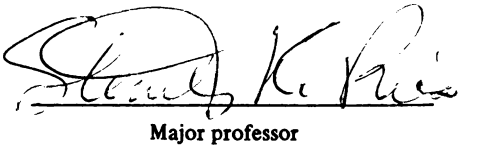
has been accepted towards fulfillment
of the requirements for

Master of Science degree in Horticulture



Major professor

Date 11-06-80

DEVELOPMENT AND CHARACTERIZATION OF AN *IN VITRO* SYSTEM RESPONSIVE TO 1-TRIACONTANOL

By

Robert L. Houtz

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1980

ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF AN IN VITRO SYSTEM RESPONSIVE TO 1-TRIACONTANOL

By

Robert L. Houtz

A system employing extracts from the leaves of rice (Oryza sativa L.) and corn (Zea mays L.) seedlings was developed for the study of the in vitro effects of triacontanol (TRIA) on plant metabolism. The system is characterized by increases in total reducible nitrogen (total N) and reducing sugars in water extracts from TRIA treated leaves. Nitrate concentrations were not affected by TRIA in this system.

Studies on the effect of pH, temperature, fractionation by centrifugation and incubation media established that the response is maximized in complete media at a pH of 6.0 and temperature of 25 to 35 C. The increases in total N due to TRIA in the crude extract of rice was doubled by centrifugation at 4080 g.

Starch phosphorylase activity in leaf segments from corn seedlings was increased approximately 40% over controls within 20 minutes after TRIA treatment. The in vivo uptake of P_i was greater in TRIA treated rice seedlings 24 hours after treatment.

ACKNOWLEDGEMENTS

I would sincerely like to thank my major professor Dr. Stanley K. Ries for his help and advice during the completion of this thesis. The knowledge and techniques of scientific investigation that I have learned from Stan are invaluable and I am sure that I will benefit from them for years to come.

I would also like to thank my committee members Dr. M. Zabik, Dr. D. Dilley, and Dr. K. Schubert for their advice and time that they contributed.

Thanks also go to Mrs. Violet Wert whose expertise in analytical techniques helped me through many tough times during my program and to my parents who made it all possible.

Finally I wish to express my thanks to Rick Knowles for his encouragement and advice during my program and to Pam Polhemus for her patience while I completed my thesis and especially her friendship.

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INTRODUCTION

1-Triacontanol ($CH_3(CH_2)_{28}CH_2OH$) (TRIA) is a 30-carbon straight chain primary alcohol with a molecular weight of 438.8 and a melting point of 88 C (Handbook of Chemistry and Physics, 57th edition, Chemical Rubber Publishing Company). TRIA is slightly soluble in ethanol, very soluble in ether and the calculated solubility in water is 6.67×10^{-37} M as determined from the formula $\log M = -1.39\ m + 5.53$ where M equals molarity and m equals the number of carbon atoms in any primary alcohol. This formula is based on the solubility of n-aliphatic alcohols in water which was determined by surface tension measurements (23). The logarithm of the solubility is a linear function of hydrocarbon chain length.

The effects of TRIA and some of the regulating parameters have been well established on intact plants (3,8,24, 44,45). The most notable are increases in dry weight, soluble protein, water uptake and total reducible nitrogen (total N) along with an apparent regulatory role of CO_2 in the atmosphere. Increases in free amino acids and total N have been measured within 40 to 80 minutes (8,24). Growth analysis, analytical studies of metabolism and some biochemical research failed to elucidate the mode or site of TRIA action. The ambiguous nature of these observations has prevented the development of a coordinated hypothesis.

The first effects of TRIA probably occur at a subcellular level and are manifested later in the ontogeny of the plant. These effects become self-limiting and may not be observed after they are delineated in whole plants. This hypothesis is supported by research with whole plants over short time periods. TRIA treated corn and rice plants had higher levels of soluble proteins, organic acids, amino acids and reducing sugars than control plants (8,3). Growth analysis of rice (Oryza sativa L.) seedlings treated with TRIA revealed an early increase in net assimilation rate (NAR) followed by a return to the control NAR (44). Studies conducted with rice leaf segments and whole plant suggest that carbohydrate pools within the plant are required for the TRIA response (14,3). The most substantial evidence for a biochemical effect at the molecular level is the rapid incorporation of deuterium oxide into the organic acid pool in TRIA treated rice plants (8).

This study was conducted to develop and characterize an in vitro system responsive to TRIA with the hope of identifying a precedent biochemical effect. Through this approach it may be possible to define a mode of action or at least demonstrate a consistent in vitro response similar to that observed in whole plants.

LITERATURE REVIEW

TRIA in Plant Cuticles.

The plant cuticle contains a variety of wax components, the most common of which are ketones, diols, adehydes, secondary and primary alcohols (25). TRIA is an example of the latter category. TRIA was first isolated from the leaves of alfalfa (Medicago sativa L.) in 1933 (6). Since then TRIA has been found to be a constituent of the free alcohols in plant waxes in a number of species in quantities from 95% to less then 1% (26,54). The location of long-chain aliphatic plant cuticular compounds was at first believed to be only in the cuticle, but waxes and fatty acids of a similar nature have been found in chloroplasts and fat bodies within leaf cells (48). The function of the cuticle is the regulation of water loss, nutrient loss by leaching, and protection against insects and fungi by its physical nature (25). The growth regulating properties of the chemical constituents of the cuticle have received little attention.

A growth promoting substance isolated from an alcoholic extract of tobacco (Nicotiana tabacum L.). 'Maryland Mamooth' leaves increased growth as measured by the oat internode bioassay (7). This same fraction demonstrated chemical properties characteristic of long-chain primary alcohols containing between 22 and 28 carbon atoms (56). A

chloroform extract from alfalfa meal was shown by Ries et al. (45) to increase the dry weight and water uptake of several plant species, and the biologically active component was identified as TRIA by mass spectrometry. Other long chain alcohols containing 8 to 11 carbon atoms inhibit or kill axillary and terminal bud growth in tobacco plants (5,50). Alcohols of longer carbon length (16-28) can selectively inhibit the increase in dry weight and water uptake ascribed to TRIA (19). Short chain alcohols ranging from methanol to butanol in concentrations less than 10^{-3} M exert a positive growth action on elongation of isolated wheat (Triticale hexaploide L.) roots (13).

Biological Activity of TRIA and other Lipids.

There are several different classes of lipids, however, all can be characterized as water-insoluble organic molecules that can be extracted from cells and tissues by non-polar solvents. Some biological functions ascribed to lipids include components of membranes, sources of metabolic fuel, protective coating on the surface of organisms and hormonal activity. The latter function of lipids has been intensely studied and characterized in mammalian systems while research in plant systems leaves much to be uncovered about the possible regulatory roles of lipids in plant growth and development.

Fatty acid esters affect the response of excised pea (Pisum sativum L.) epicotyls to applied auxin (51). Methyl linoleate and methyl oleate in the pea bioassay apparently

increased the sensitivity of the pea sections to IAA, implicating a close relationship between auxin activity and lipid metabolism (51). Homologous series of alkyl chlorides, bromides and iodides enhanced auxin-induced pea stem elongation at levels of 3 to 40 micromolar, the molecular length of these lipids was the determining factor for activity (52). In all the cases investigated using the pea stem elongation bioassay, lipids stimulated respiration in addition to augmenting cell elongation. Certain synthetic aliphatic hydroxycarboxylic acids have shown growth promoting activity on the root growth of lettuce (Lactuca sativa L.) seedlings (12).

Unsaturated fatty acids have a number of effects on isolated mitochondria and chloroplasts. Exogenous unsaturated fatty acids such as oleic or linolinic suppress the Hill reaction in isolated chloroplasts (35). This suppression was associated with a visable swelling of the chloroplast. Later investigation of the swelling suggested that the association of the fatty acids with the chloroplast membrane results in a decreased rigidity and polarity of the membrane (39,48).

TRIA was shown in have growth regulating activity by Ries et al. (45) in 1977. In experiments with several crop species, dry weight and water uptake were increased over controls by treatment with TRIA. Although the first studies were conducted over 7 to 8 days, later work indicated that the effect of TRIA occured within the first 24 h period after treatment (44). Increases in protein and leaf area were also observed but the most prodigious effect was a dry weight

increase within 6 h in the dark where controls typically lost dry weight. At first it was thought that the dry weight increase in the dark was due to TRIA stimulation of dark CO_2 fixation. However, later studies by Bittenbender et al. (3) showed that there was no net dark CO_2 fixation in response to TRIA treatment but CO_2 played a regulatory role rather than substrate in the TRIA response. Increase in respiration, soluble and insoluble Kjeldahl-N and soluble carbohydrates were also reported. It was postulated that this increase in dry weight was due to the metabolic incorporation of water through hydrolysis of some stored products within the plant.

TRIA increased the growth of cell cultures of several crop species apparently by increasing the cell number (14). Tests with octacosanol [$CH_3(CH_2)_{26}CH_2OH$] suggested that a specific chain length may be required for activity (45). This hypothesis was substantiated by Jones et al. (19), when the activity of several analogs of TRIA were tested for growth promoting effects on rice seedlings. Perhaps more important was the finding that all the analogs and other long chain hydrocarbons applied in combination with TRIA at equimolar concentrations inhibited the TRIA response in rice and tomato seedlings.

Analysis of ^2H incorporation with deuterium oxide into plant metabolites in control and TRIA treated rice seedlings using a stable isotope tracer technique and metabolic profiling, indicated rapid increases in the pools of succinate and some α-amino acids in TRIA treated plants (8). The results insinuate that the effect of TRIA is related to the

control of the level of organic nitrogen available for protein synthesis in the leaves. The carbon used to increase the α-amino acid pools in 10 min was believed to arise from glucose metabolism rather than from photosynthesis (8).

To date only one enzyme has been shown to be affected by TRIA. Both dark and light-grown lettuce leaf tissue exhibited greater polyphenol oxidase activity than controls when treated with TRIA (16). In studies on seed germination and early growth in 15 species, TRIA had no effect on germination or morphology (17).

Plant age and concentration of TRIA have been shown to be important factors in obtaining a response (47). The growth of several vegetable and field crops was increased under greenhouse conditions by applications of TRIA to the foliage, soil, or seed (41). However, in the field only foliar sprays increased the yields of 7 out of 10 crops tested.

The most recent work on the TRIA response in rice seedlings has shown that TRIA treatment results in an apparent increase in Kjeldahl-N that can be observed in as little as 40 min (24). However, studies with ^{15}N-enriched and depleted plants revealed that no change occurred in atom % ^{15}N. It was concluded that the increase in total N was an artifact of Kjeldahl analysis unique to TRIA treated plants, TRIA therefore must have initiated a gross change in the plants chemistry altering the Kjeldahl analysis (24). These conclusions were reached after the majority of the studies that follow had been completed.

MATERIALS AND METHODS

Plant Culture.

Rice seed, 'IR-8', 'ESD 7-1' and 'Starbonnet', were surface sterilized with 0.1% (w/v) $HgCl_2$ and germinated in 77 ml plastic cups containing vermilculite and turface (Wyandotte Chem. Co., Detroit, MI). The plants were grown as previously described (43).

Field corn 'Pioneer 3780' was planted in 18 cm clay pots in a 1:1:1 (v/v/v) sterilized mix of peat, sand and sandy loam soil. Greenhouse conditions were maintained at 25 C night temperature. Seven days after sowing, the seedlings received 250 ml of a 20-20-20 soluble fertilizer at 1.0 g/l twice weekly.

Chemical Treatment Applications.

Pure synthetic TRIA was used for all treatments (American Cyanamide, Princeton, New Jersey). TRIA treatments were prepared from either a TRIA-Tween 20 (polyoxyethylene sorbitan monolaurate) or a TRIA-chloroform stock solution, 0.1 to 1.0 mg/ml or 1.0 mg/ml respectively. The stock solutions were diluted with double distilled deionized water to give a solu- of the desired concentration. Treatment solutions made up from the chloroform stock were placed on a warm stirring plate until the chloroform droplets had disappeared. Those solutions

made up from TRIA-Tween 20 stock contained 0.1% (w/v) Tween 20. All solutions used in controls were prepared exactly as the TRIA solutions minus the TRIA.

The fourth and fifth leaves from 18 to 22 day old rice plants or the third and fourth leaves of 8 to 10 day old corn plants were excised and weighted. The leaves were immediately placed in an Erlenmyer flask containing 250 ml of treatment or control solution per g fresh weight of leaves and shaken for one min. After removal of the leaves from the treatment solution they were extracted.

For the starch phosphorylase (EC 2.4.1.1) studies a similar procedure was used except corn leaves were cut into 1 cm segments after treatment and incubated in 20 mM potassium phosphate buffer (pH 7.0) for 20 min before extraction of the enzyme.

Extraction Procedure.

Treated rice leaves were extracted at 4 C with cold 20 mM potassium phosphate buffer (pH 7) containing 1.0 mM β-mercaptoethanol, in the ratio of 7 ml buffer/g fresh weight. Corn leaves were extracted with the same buffer and conditions except 4 ml/g fresh weight was used. Rice leaves were homogenized in a cold room with an automatic mortar and pestle (Torsion Blaance Co., Clifton, New Jersey). Corn leaves were homogenized by hand with a cold mortar and pestle held on ice. The crude extract was used for the initial *in vitro* test or centrifuged at 4080 g at 4 C for 20 minutes to obtain the supernatant fraction. The supernatant suspension was poured

into a cold beaker held on ice and stirred. Aliquots were pipetted from this solution.

Starch phosphorylase was extracted from corn leaves with cold 10 mM maleate-KOH buffer (pH 6, 4 ml/g fresh weight) by grinding in a cold mortar and pestle (4 ml/g fresh weight). The extract was filtered through four layers of cheesecloth and centrifuged at 20,000 g for 20 min. The supernatant fluid was then used as the enzyme source.

Incubation Parameters.

The crude extract or the supernatant fraction was added to cold incubation media in a ratio of 1:4 (v/v) in 50 ml or 125 ml Erlenmyer flasks on ice. Incubation media was made up fresh the day of the experiment with the same buffer used for extraction and contained the followed components: NADPH 1.3 mM, NADH 0.13 mM, ATP 0.18 mM, $MgCl_2 \cdot 6\ H_2O$ 0.28 mM, oxaloacetic acid 0.13 mM, ∝ketoglutarate 0.12 mM. All of the above reagents except magnesium chloride hexahydrate were obtained from Sigma Chemical Co., St. Louis, Missouri, the $MgCl_2 \cdot 6\ H_2O$ was analytical grade.

Initial in vitro experiments with the crude extracts were carried out with a reciprocating shaker that was placed in a growth chamber and held at 25 C for the duration of the experiment. Light conditions were the same as that used for the growth of the rice seedlings. Incubation of the supernatant fraction was conducted in a reciprocating water bath shaker held at 25 C on a lab bench.

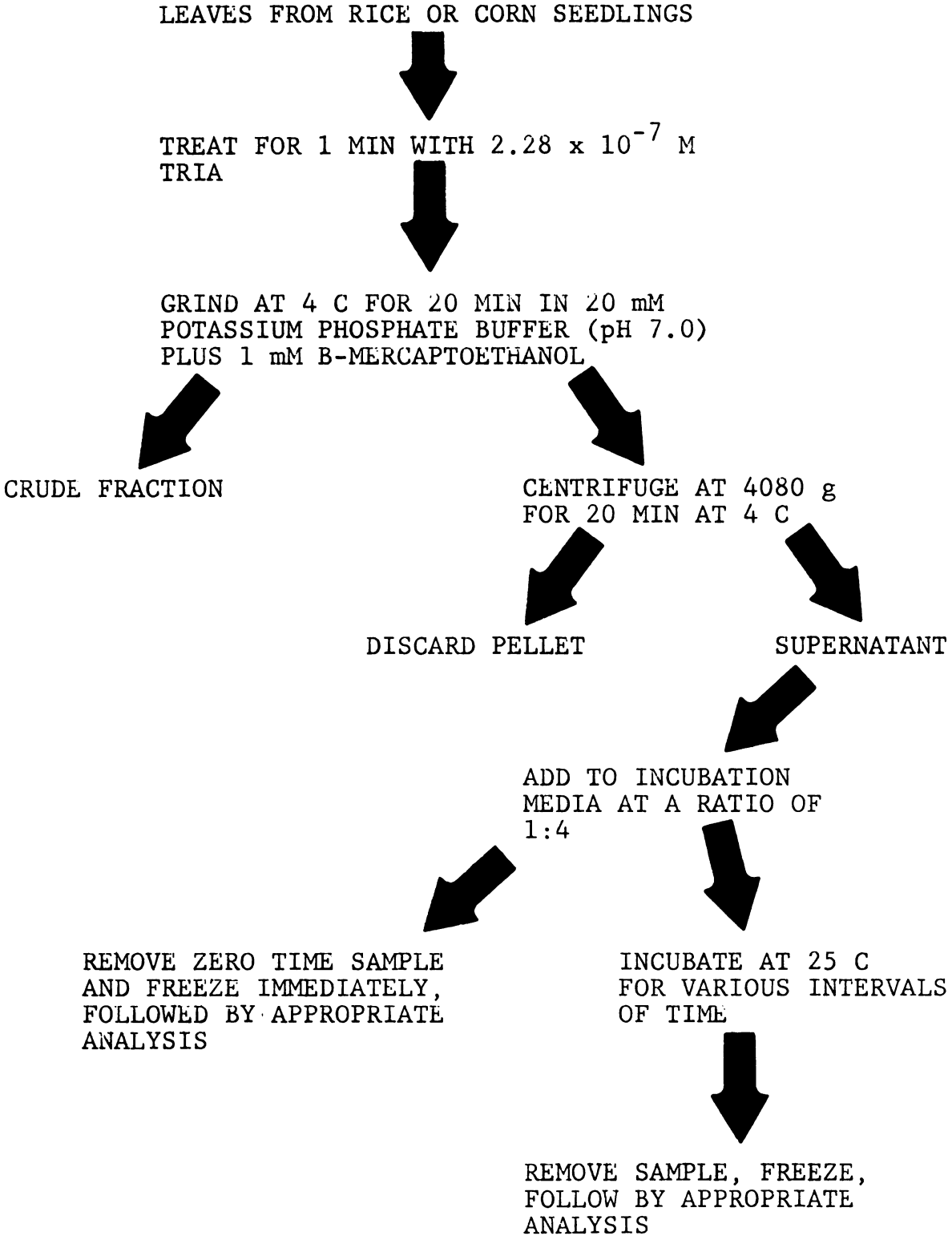
After addition of the extract to the cold incubation media, samples were removed for zero time analysis and the remainder placed on the shaker. Additional samples were removed at the designated time for analysis. A simplified schematic is shown (Figure 1) depicting the extraction and incubation parameters used.

Starch phosphorylase activity was determined by measuring the rate of P_i production. The reaction mixture consisted of 10 mM maleate-KOH buffer, 0.3% soluble starch (w/v) (Difco Laboratories, Detroit, MI), 50 mM glucose-1-P (Sigma) and enzyme in a total volume of 2 ml. The reaction mixture without enzyme was placed in a water bath shaker held at 37 C and after addition of the enzyme the P_i liberated was measured over 10 to 20 min intervals by the method of Taussky and Shorr (53).

Sampling and Analysis Methods.

Three ml samples were used for all total N determinations and 1 ml samples for all other analyses. The samples for total N were pipetted into 50 to 125 ml Erlenmyer flasks and frozen with a mixture of dry ice and acetone. After freezing the samples were either lyophylized, dried at 65 C in a forced air over or digested immediately with the digestion mixture used for Kjeldahl digestion. All total N was determined by an automated micro-Kjeldahl procedure (10). Samples for total N determination were digested with a solution containing 1800 ml concentrated H_2SO_4, 40 ml 70% $HClO_4$, 160 ml distilled water and 6 g SeO_2. Four mls of the

Figure 1. Procedure for isolation of crude and supernatant fractions from rice or corn seedlings used for the study of the in vitro effects of TRIA.


LEAVES FROM RICE OR CORN SEEDLINGS
TREAT FOR 1 MIN WITH 2.28 x 10-7 M TRIA
GRIND AT 4 C FOR 20 MIN IN 20 mM POTASSIUM PHOSPHATE BUFFER (pH 7.0) PLUS 1 mM B-MERCAPTOETHANOL
CRUDE FRACTION
CENTRIFUGE AT 4080 g FOR 20 MIN AT 4 C
DISCARD PELLET
SUPERNATANT
ADD TO INCUBATION MEDIA AT A RATIO OF 1:4
REMOVE ZERO TIME SAMPLE AND FREEZE IMMEDIATELY, FOLLOWED BY APPROPRIATE ANALYSIS
INCUBATE AT 25 C FOR VARIOUS INTERVALS OF TIME
REMOVE SAMPLE, FREEZE, FOLLOW BY APPROPRIATE ANALYSIS

digestion mixture were added to each dried 3 ml sample from the in vitro experiments or directly added to 3 ml liquid samples. The samples were heated until the solution cleared and cooled, followed by addition of 4 or 1 ml distilled water for dried or liquid samples respectively. The samples were then placed on an Auto-Analyzer (Technicon Instruments Corporation, Tarrytown, NY) for sampling and automated total N analysis by reacting the NH_4^+ ions in the acid mixture with alkaline phenol and NaOCl. The reaction generates a blue color with maximum absorbance at 632 nm. Standards were prepared from ground wheat (2.566% N) and all data calculated from a regression line computed from the wheat standards. The percent nitrogen in the ground wheat was calibrated using the nitrogen standard for plant material from the National Bureau of Standards, Washington, D. C. (standard reference material 1571).

One ml samples from which nitrate, reducing sugars, soluble carbohydrates, soluble protein and free amino acids were quantified were removed from the incubating extracts at various times, placed in 1 x 10 cm test tubes and quick frozen. The samples were then held at -70 C for not more than 48 h until the appropriate analysis could be performed. All spectrophotometric determinations were carried out using triplicate samples from the thawed extracts.

For nitrate analysis 20 µl samples were removed from the rice extracts or 10 µl samples from corn extracts and added to 0.5 ml of 0.1 M K-succinate buffer at pH 6.8 in 1 x 10 cm test tubes. The nitrate was then determined by the method of

Lowe and Hamilton (30). The concentration of nitrate in the sample was calculated from a linear regression line generated from a set of standards prepared from a stock KNO_3 solution (12.4 µg/ml).

Samples of 50 µl each for corn and rice extracts were used for determination of reducing sugars. The samples were placed in 2 x 15 cm test tubes containing 1 ml deionized distilled water and the reducing sugars determined by the method of Nelson and Somogyi (38,49). Standards were prepared from an ∝-D(+) glucose solution (1 mg/ml) and all data calculated from a linear regression line on the standards.

Free amino acids were determined by a modified ninhydrin colorimetric procedure developed by Rosen (46), using 50 µl samples from both corn and rice extracts. All data was calculated from a linear regression line using leucine as a standard (1 µmole/ml).

Total soluble carbohydrates analysis was carried out on 50 µl samples from the rice and corn extracts by the phenol-sulfuric acid method as described by Hodge and Horfreike (18) and all data obtained from a linear regression line on a set of standards prepared from a ∝-D(+) glucose solution (1 mg/ml).

Soluble protein determinations were carried out on 10 µl samples from both corn and rice extracts using a modification of Lowry (31). The modification is as described by Bensadoun and Weinstein (1) and effectively removes many of the substances present in plant extracts that interfere with the normal Lowry procedure. Bovine serum albumin was used as the

standard (1 mg/ml) and all data calculated from a linear regression line on the standards.

For starch phosphorylase activity of the P_i production in the reaction mix was measured by removing 100 µl samples over 10 or 20 min intervals and precipitating the protein with 4 ml of 10% (w/v) trichloracetic acid. The mixture was well agitated and then centrifuged at 1000 g for 30 min. The supernatant fluid was removed and 3 ml used for the determination of P_i. Standards were prepared from a stock potassium phosphate solution (1.0 mg/ml) and all data calculated from a linear regression line on the standards and blanks consisted of samples from the reaction mix minus enzyme. Blanks were also prepared from reaction mix minus soluble starch so that any increase in P_i due to phosphatase activity could be subtracted from the starch phosphorylase activity data. Protein levels in the extracts used for this assay were determined by the same modification of Lowry described earlier.

Statistical Analysis.

Several designs were used. Most often a randomized complete block was used blocking for position in the incubator. Where it was appropriate trend analysis was conducted. All data was subjected to analysis of variance and the means compared by use of the least significant difference except where there was only one degree of freedom for the treatment. In these instances the F value from the analysis of variance was used for comparison of means. Starch phosphorylase data was

analyzed by linear regression. There was very little variation in these tests indicated by range of coefficients of variations of 0.5% to 2.0% for all of the experiments. Error due to pipetting could account for only 2.6 µg nitrogen/ml while the detection limit of the automated micro Kjeldahl is approximately 4 µg nitrogen.

RESULTS AND DISCUSSION

Total N Response Studies.

One of the most rapid and significant changes in TRIA treated plants is the increase in total N (8,24). This response can be detected within 40 min (8, unpublished data, Ries). The results of a preliminary test with a crude extract from TRIA treated rice leaves revealed that a similar total N response could be observed in vitro (Table 1, Test 1). The total N in the TRIA treatment increased from zero time to 320 min. There was no change in total N in controls treated with a solution of 0.1% Tween 20. In a second test the extract from TRIA treated leaves again demonstrated an increase in total N over an 320 min interval, but the extract from leaves treated with 0.1% Tween 20 showed no change in total N over time (Table 1, Test 2).

Method of Treatment

Experiments were conducted to determine if TRIA could be added directly to the extracts or through the extraction buffer during extraction and the same response still be observed. Addition of the extract to incubation media containing all components plus 1 mg/l TRIA had no significant effect on the total N content in the extract. In this

Table 1. Total N levels in crude extracts from TRIA-treated rice leaves over time. Each treatment in each test contained 4 ml crude extract and 8 ml incubation media in 125 Erlenmeyer flask, after the appropriate times the extracts were quick frozen and dried at 65 C. After drying the entire residue was digested for total N determination. Each mean is the average of 10 replicates.

Treatments		Test 1	Test 2
Time (min)	TRIA (100 µg/l)	Nitrogen (mg/flask)	
0	0		2.09
0	+	1.87	1.97
20	0		1.98
20	+	1.99	2.08
80	0		1.98
80	+	2.05	2.10
320	0		1.96
320	+	2.05	2.15
L.S.D. at .01 level		0.15	0.12

experiment with 6 replicates the average zero time and 80 min total N values in the extract were 836 and 832 µg N/ system ± 2.88, respectively.

Addition of TRIA (1 mg/l) via the extraction buffer also was ineffective in eliciting an increase in total N. The average zero time and 80 min total N value from 8 replicates in this experiment were 754 and 755 µg N/system ± 2.55 respectively.

These experiments suggest that the integrity of the plant tissue must be maintained during treatment with TRIA for an *in vitro* effect to be observed, but once TRIA has reached its active site the initiation of the response is achieved and disruption of the tissue from this point does not result in a loss of activity.

Centrifugation Experiments.

Centrifugation of the crude extract at 4080 g doubled the observed difference in total N between the zero time and 80 min treatments (Table 2). The augmentative effect of centrifuging the extract could have been due to the removal of impurities or inhibitors of the factor(s) responsible for the total N increase. The response in this supernatant fraction was found to be linear over time up to 80 min, a control supernatant suspension indicated no change in total N as had been observed earlier with the crude extracts (Figure 2).

A similar experiment with the supernatant fraction from leaves of 9 day-old corn plants revealed a trend similar to

Table 2. Response of crude and supernatant fractions from rice leaves treated with TRIA (100 µg/l). Each mean is the average of 8 replicates.

Extract Fraction	Time (min)	Total N (mg/ml)
Crude	0	.318
	80	.333*
Supernatant (4080 x g's)	0	.212
	80	.245**

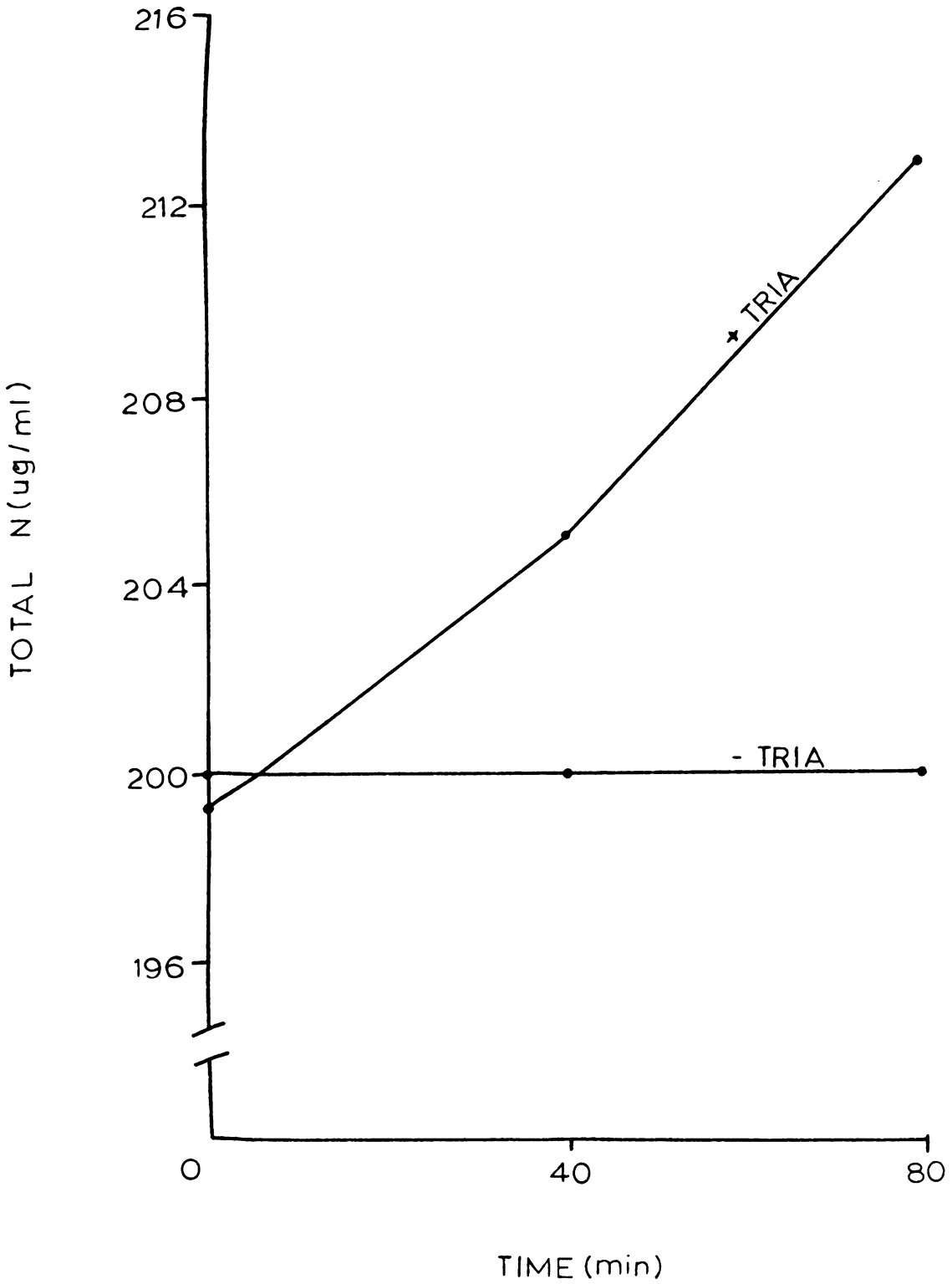
*,** F value for comparison with zero time significant at .05 and .01 levels, respectively.

that observed in the rice extracts (Table 3). In this experiment nitrate nitrogen was also determined and the possibility that the in vitro reduction of nitrate by nitrate reductase was responsible for the total N increase was rejected. Latter experiments with rice extracts will support the same conclusion for rice. This data agrees with studies conducted on the effect of TRIA on the enzymatic reduction of nitrate nitrogen (24). Dry weights of the supernatant were obtained at the beginning of the experiment and all subsequent total N and nitrate data expressed per g dry weight.

Further investigation of the effect of centrifugation on the total N response in rice and corn extracts from TRIA treated leaves revealed no increase in the magnitude of the response with increasing centrifugation force (Table 4).

The increased response to TRIA from centrifugation at 4080 g with no enhanced activity from further centrifugation

Figure 2. Total N concentration with time in the supernatant fraction (4080 g's) from rice leaves treated with 0.1% Tween 20 or 0.1% Tween 20 + 100 μg/l TRIA. The F value is significant at the .01 level for the interaction of TRIA x linear time. Each mean is the average of 10 replicates.


216
212
208
204
200
196
TOTAL N (ug/ml)
+ TRIA
- TRIA
0
40
80
TIME (min)

suggest that the site of TRIA action is in the soluble fraction of the plant cell. The insoluble portion at most

Table 3. Total N and nitrate levels in the supernatant fraction from corn leaves. Each mean is the average of 6 replicates

Treatments		Nitrogen (mg/g dry weight)	
TRIA (100 µg/l)	Time (min)	Total (Kjeldahl)	Nitrate
+	0	74.70	3.7
+	30	81.45**	3.8
+	60	81.80**	3.7
+	120	82.93**	3.7
+	1380	75.93	3.8

** F Value for comparison with zero time significant at .01 level.

Table 4. Effect of centrifugation force on total N increase in extracts from TRIA treated (100 µg/l) leaves. Each mean is the average of 9 replicates.

Time (min)	Centrifugation force (xg)	Total N (µg/ml)	
		Rice	Corn
0		328	297
60	4000	334**	305**
0		305	286
60	8000	311**	294**
0		289	277
60	16000	296	286**

Table 4 (cont'd)

** F value for comparison with zero times significant at .01 level.

plays a minor role in the TRIA response *in vitro*.

Incubation Media Studies

Experiments were conducted in order to test the necessity of the incubation media for the TRIA response. After centrifugation of the crude extract, at 4080 g's the supernatant solution was added to buffer at a ratio of 1:2, zero time samples removed and the extract incubated at 25 C. After 60 min, 3 ml samples were removed and the total N determined as described under Materials and Methods. Removal of the components of the incubation media from the extract eliminated the increase in total N for corn and rice leaves (Table 5). Further investigation into the role of the

Table 5. Effect of incubation media on total N increase in extracts from TRIA (100 µg/l) treated leaves. The total N values for + incubation media have been corrected for the nitrogen in the incubation media. + and 0 incubation media were separate experiments. Each mean is the average of 10 replicates

Time (min)	Incubation Media	Total N (µg/ml) Rice	Corn
0	+	205	274
60	+	220**	282**
0	0	220	276
60	0	220	279

Table 5 (cont'd)

** F value for comparison with zero time significant at .01 level.

components of the incubation media was conducted with the supernatant from rice leaves. The incubation media components were removed separately in groups of similar chemical and metabolic nature. Treatments were included where none or all of the components were removed. Removal of any one group eliminated in the response (Table 6). In some tests the removal of α-ketoglutaric acid and oxaloacetic acid had no effect on the total N increase, so the necessity of the two carbon skeletons still remains obscure even though on the average they are apparently necessary. Apparently for TRIA to elicit a total N increase *in vitro* reducing power and biological energy are required in the form of reduced pyridine nucleotides and adenosine 5'-triphosphate.

Effect of TRIA on Reducing Sugars in vitro.

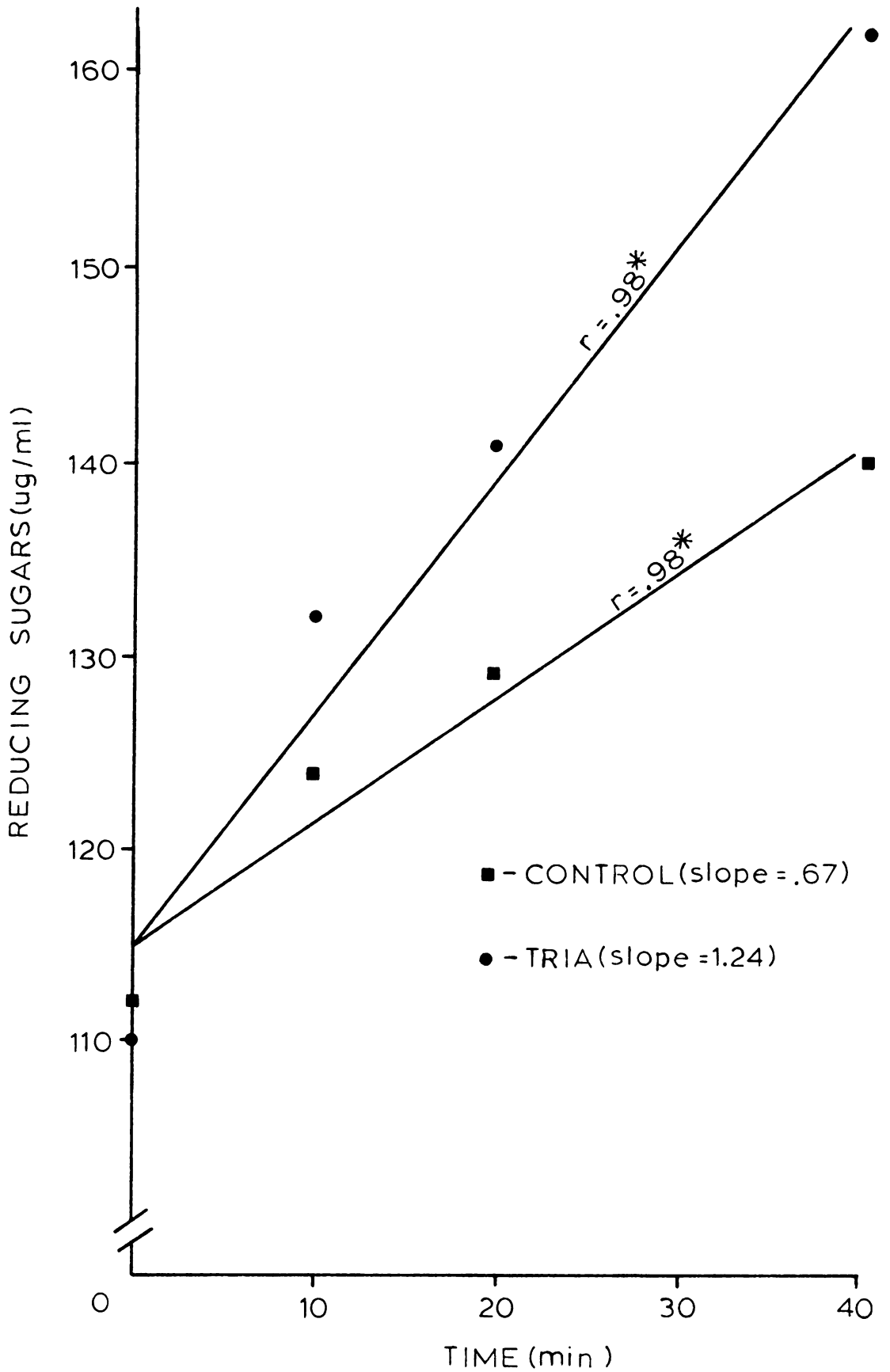
Since several earlier studies on the effect of TRIA in whole plants implicated the involvement of carbohydrate metabolism in the TRIA response (3,8,14), experiments were conducted to determine if a similar response was occurring in vitro. When extracts from TRIA and control treated rice leaves were incubated at 35 C, the level of reducing sugars measured at 0, 10, 20 and 40 min increased linearly with both control and TRIA treatments (Figure 3). However, the rate of increase in reducing sugars with TRIA treatment was almost twice as large as the rate with control treatments. These

Table 6. Effect of components of incubation media on the total N increase from TRIA (100 µg/l) treated rice leaves. Each mean is the average of 10 replicates.

Component removed	Time (min)	Total N (µg/ml)
None	0	278
	60	284**
NADH, NADPH	0	280
	60	274
ATP, $MgCl_2$	0	281
	60	277
OAA, ∝KETO	0	281
	60	284
ALL	0	300
	60	301

** F value for comparison with zero time significant at .01 level.

Figure 3. Level of reducing sugars in supernatant fraction from rice leaves incubated at 35 C. Each mean is the average of 4 replicates.
* r value significant at .05 level for linear regression


160
150
140
130
120
110
0
10
20
30
40
REDUCING SUGARS (ug/ml)
TIME (min)
r = .98*
r = .98*
■ - CONTROL (slope = .67)
● - TRIA (slope = 1.24)

results suggested that TRIA treatments were facilitating the rapid hydrolysis or breakdown of carbohydrates in the extract. Since lower night temperatures during the development of some plants leads to high starch levels within the leaves, an experiment was conducted with corn plants raised under 16 C and 10 C night temperatures with a day temperature of 30 C. The level of soluble carbohydrates was higher at 10 C than at 16 C and the increase in total N was also greatest with plants grown under 10 C nights (Table 7). The plants used for the

Table 7. Total N and soluble carbohydrate levels in extracts from corn leaves treated with TRIA (100 µg/l) as affected by night temperature. 10 C and 16 C are separate experiments. Each mean is the average of 8 replicates.

Night temperature	Time (min)	Soluble carbohydrates (µg/ml)	Total N (µg/ml)
	0	870	170
16 C	60	879	173*
	0	1419	312
10 C	60	1441	317**

*,** F value for comparison with zero time significant at .05 and .01 levels, respectively.

10 c night temperature were 14 days old and the plants for the 16 C 11 days old but the heat units accumulated prior to treatment were equal.

Reducing sugars were found to increase significantly in extracts from TRIA treated corn leaves (Table 8). Again nitrate determinations were carried out to ensure that the

Table 8. Nitrate, reducing sugars and total N levels in extracts from TRIA (100 µg/l) treated corn leaves. Each mean is the average of 8 replicates.

Time (min)	Nitrate	Reducing sugars	Total N
		(µg/ml)	
0	126	664	285
60	124	719*	292**
120	121	728*	293**

*,** F value for comparisons with zero time significant at .05 and 0.1 levels, respectively.

observed total N increase was not due to the in vitro reduction of nitrate. No significant decrease in nitrate nitrogen was observed.

Effect of pH and Ratio of Extract to Incubation Media.

Two other parameters hypothesized to play an important role in the in vitro TRIA response were pH and the ratio of extract to incubation media. If the TRIA induced increase in total N and reducing sugar observed in vitro is enzymatic then doubling the level of extract in the incubation system should double the response during short time intervals. When two times the normal amount of supernatant solution from TRIA treated rice leaves was added to incubation media that had been prepared in half the normal volume of buffer used, the total N increase was doubled (Table 9), however, the increase in reducing sugars was quadrupled.

This data suggests that if an enzyme or substrate is involved in the total N response due to TRIA it must have

Table 9. Reducing sugars, nitrate and total N levels as affected by time and ratio of extract incubation media in extracts from TRIA (100 µg/l) treated rice leaves. Each mean is the average of 8 replicates.

Time (min)	Extract concentration	Total N	Reducing sugars	Nitrate
		(µg/ml)		
0	1x	326	596	78
60	1x	330*	641*	76
0	2x	622	1208	158
60	2x	630**	1380**	160

*,** F value for comparison with zero time significant at .05 and .01 levels, respectively.

been increased by a factor of two, but both the enzyme(s) and substrate(s) responsible for the reducing sugar increase must have been doubled to give an increase of four-fold over the normal extract concentration. Therefore, the two effects attributed to TRIA observed in vitro, a total N and reducing sugar increase must be the result of two different effects of TRIA, even though they show similar trends over time.

The effect of pH on the total N and reducing sugar increase was studied by incubating the extract plus incubation media at three different pH's and measuring the total N and reducing sugar levels were affected in the same manner by increasing pH. Therefore, it appears that the increases in total N and reducing sugars may be related. However as mentioned earlier, manipulating the extract concentration had separate effects on the total N and reducing sugar increase from TRIA. An investigation of the literature revealed that

Table 10. Effect of pH on the increase in reducing sugars and total N in extracts from TRIA treated corn leaves. The total N and reducing sugar zero time values were 250 and 120 µg/ml, respectively. Each mean is the average of 10 replicates.

Time (min)	pH	Δ Total N[α] (µg/ml)	Δ Reducing sugars[α] (µg/ml)
0-60	6	10.2	240
0-60	7	7.0	176
0-60	8	5.5	114

[α] F value for the linear decrease of total N and reducing sugars with increasing pH is significant at .01 level.

total N determination by the method of Ferrari (10) can be affected by reducing sugars and in general plant carbohydrates, high concentrations of either can result in variable reduction of nitrate nitrogen thereby increasing the observed total N (4,40). At first this was believed to be the source of the increase in total N observed in extracts from TRIA treated leaves and perhaps in whole plants. However, studies on the source of the nitrogen increase in plants treated with TRIA revealed that this was not true (24). So the total nitrogen increase remained to be explained although it was now known to be an artifact associated only with TRIA treated plants.

Incubation Temperature and Inhibitor Studies.

The effect of different temperatures during the incubation of the extracts in the TRIA response in vitro was studied. The

observed increases in total N and reducing sugars were different for each 10 C increase in temperature. The increase in reducing sugars over a 60 min interval was at least doubled by each 10 C increase in temperature, however, the total N increase was less than two for each 10 C increase in temperature as shown by the Q_{10} (Table 11).

Table 11. Effect of incubation temperatures on reducing sugars and total N levels in extracts from TRIA (100 g/l) treated corn leaves. Each mean is the average of 7 replicates.

Incubation temp. (°C)	Time (min)	Total N (g/ml)	Q_{10}	Reducing sugars (g/ml)	Q_{10}
25	0	181		385	
25	60	184*		392	
35	0	180		383	
35	60	185**	1.7	405**	3.1
45	0	181		377	
45	60	187**	2.0	442**	2.9

*,** Significance at 5% and 1% levels for increase over zero time, respectively.

Octocosanol [$CH_3(CH_2)_{26}CH_2OH$] an analog of TRIA completely inhibits the TRIA response in rice and tomato plants in an unexplainable manner (19). When octacosanol was applied along with TRIA to rice leaves the supernatant fraction exhibited no significant increase in total N or reducing sugars (Table 12). Inhibition of the TRIA response <u>in vitro</u> by

octacosanol is further evidence that the *in vitro* effects of TRIA parallel the response in whole plants.

Table 12. Inhibition of increase in total N and reducing sugars by octacosanol in extracts from TRIA treated corn leaves. Each mean is the average of 10 replicates.

Time (min)	(100 µg/l)	Octacosanol (50 µg/l)	Total N (µg/ml)	Reducing sugars (µg/ml)
0	+	0	257	729
60	+	0	266**	770*
0	+	+	262	756
60	+	+	264	745

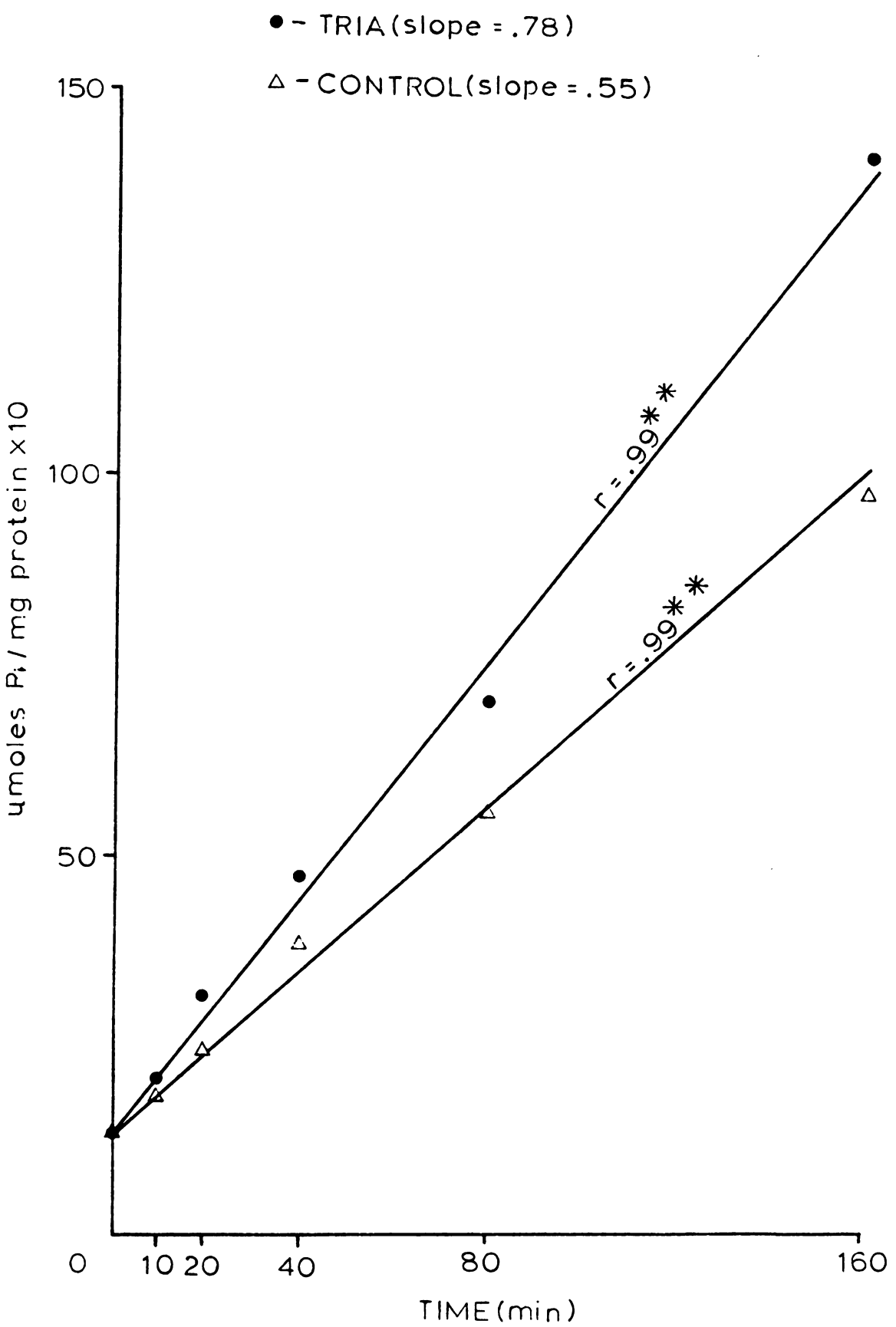
*,** F value for comparison with zero time significant at .05 and .01 levels, respectively.

Studies on Starch Phosphorylase Activity.

The increase in reducing sugars *in vitro* and previous work with whole plants lead to the hypothesis that starch phosphorylase activity may be higher in TRIA treated plants. To test this hypothesis the leaves from 9 day-old corn plants were excised, treated with TRIA (100 µg/l) and then incubated in 20 mM phosphate buffer for 20 min after which time the enzyme was extracted and assayed. In four out of four experiments the starch phosphorylase activity from TRIA treated leaves was greater than in controls (Figure 4).

It was predicted that TRIA treated plants would take up more P_i in response to the increased starch phosphorylase activity. In an experiment with whole rice plants the typical

Figure 4. Starch phosphorylase activity from control and TRIA (100 g/l) treated corn leaves. Each mean is the average of 4 replicates.
** r value significant at .01 level for linear regression.


● - TRIA (slope = .78)
△ - CONTROL (slope = .55)
150
100
50
0
μmoles Pi / mg protein X10
r = .99**
r = .99**
10 20
40
80
160
TIME (min)

increase in dry weight associated with TRIA treatment was observed but more importantly the P_i uptake was also significantly increased over the control plants after 24 h (Table 13). This was the first experiment where the results

Table 13. Inorganic phosphate, dry weight and water intake in control and TRIA treated 12-day-old rice seedlings after 24 h. Each mean is the average of 8 replicates.

TRIA (100 µg/l)	Time (h)	Water uptake (ml)	Dry wt. (mg)	Inorganic phosphate (µM)
0	0	--	34.0	354
0	24	4.9	38.0	340
+	24	5.2	40.6**	314**

** F value for comparison with controls significant at .01 level.

from in vitro studies had been used to anticipate the response of whole plants to TRIA treatment.

Starch phosphorylase activity is increased by treatment with kinetin in potato (Solanum tuberosum L.) tubers. Although phosphorylase in the mammalian system is highly regulated by 5' adenylic acid, studies on the phosphorylase from potato tissue showed no effect of 5' adenylic acid or other nucleotides (27,28). TRIA may affect starch phosphorylase activity directly or indirectly through a change in the enzyme environment before isolation. It has been shown that binding sites exist on potato phosphorylase that will accept hydrophobic groups of a saturated nature six carbons long (37). Perhaps through one of these sites TRIA could stimulate a

change in the conformation of the enzyme resulting in increased activity although there is no evidence for this.

CONCLUSIONS

The apparent total N increase observed in whole plants treated with TRIA may be measured *in vitro*. The increase in total N is apparently dependent upon the presence of reduced pyridine nucleotides and adenosine 5'-triphosphate. The increase was maximized when the plants used in this *in vitro* system were grown under a 30 C:10 C day:night temperature regime which resulted in a high level of total soluble carbohydrates. A linear increase in reducing sugars above that in controls was evident *in vitro*. This effect is unique to the *in vitro* system. The total N increase reflects some enzymatic properties but not wholly, while the reducing sugar increases fulfills several of the requirements for an enzyme catalyzed reaction.

Octacosanol an effective inhibitor of the TRIA response in whole plants works equally well at eliminating the total N and reducing sugar increases *in vitro*. Previous work on the enzymatic reduction of nitrate nitrogen as affected by TRIA treatment lead to the conclusion that there was no effect of TRIA on this enzyme, or that increase in total N observed in whole plants originated by this reaction. This conclusion is substantiated *in vitro* where the nitrate levels showed no significant decrease over time.

Several earlier reports on the growth promoting effects of TRIA had implicated carbohydrate hydrolysis as a possible precursor to many of the effects observed at longer periods of time after treatment. Starch phosphorylase activity is shown here to be on the average approximately 40% greater than that in controls. Extrapolation back to whole plants lead to the discovery that the inorganic phosphate uptake in rice seedlings is increased by treatment with TRIA. The TRIA response in vitro is similar to that observed in whole plants. More important is the finding that an enzyme involved in carbohydrate metabolism is affected by TRIA. It is possible that this is the precedent effect of TRIA on plant metabolism and that the responses previously ascribed to TRIA are of a secondary nature. However, more research is required for conclusive evidence in regards to this hypothesis.

LITERATURE CITED

LITERATURE CITED

1. Bensadoun, A. and P. Weinstein. 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. 70:241-250.

2. Bieleski, R. L. 1973. Phosphate pools, phosphate transport, and phosphate availability. Ann. Rev. Plant Physiol. 24:225-252.

3. Bittenbender, H. C., D. R. Dilley, V. Wert and S. K. Ries. 1978. Environmental parameters affecting dark response of rice seedlings to triacontanol. Plant Physiol. 61: 851-854.

4. Bremmer, J. M. 1960. Determination of nitrogen in soil by the Kjeldahl method. J. Agric. Sci. 55:11-33.

5. Cathey, H. M., G. L. Steffens, N. W. Stuart and R. H. Zimmerman, 1966. Chemical pruning of plants. Science 153:1382.

6. Chibnall, Charles Albert, E. F. Williams, A. L. Latner and S. H. Piper. 1933. The isolation of n-triacontanol from lucerne wax. Biochem. J. 27:1885-1888.

7. Crosby, D. G. and A. J. Vlintos. 1959. Growth substances from Maryland Mammoth tobacco: long chain alcohols. Contrib. Boyce Thompson Inst. 20:283-292.

8. DeWitt, D. L., C. C. Sweeley, J. F. Jones and S. K. Ries. 1979. Analysis of ^{2}H incorporation into plant metabolites in control and 1-triacontanol-treated rice seedlings. Proc. Third Int. Conf. on Stable Isotopes. Argonne Nat. Lab., Chicago, IL, pp. 253-265.

9. Fekete, M. A. R. 1968. The role of phosphorylase in starch metabolism in plastids. Planta (Berl.). 79: 208-221.

10. Ferrari, A. 1960. Nitrogen determined by a continuous digestion and analysis system. N.Y. Acad. Sci. 87: 792-800.

11. Giaquinta, R. T. 1979. Phloem loading of sucrose. Plant Physiol. 63:744-748.

12. Gross, D. 1975. Growth regulating substances of plant origin. Phytochemistry 14:2105-2112.

13. Gudjohsdottir, S. and H. Burstrom. 1962. Growth-promoting effects of alcohols on excised wheat roots. Physiol. Plant 15:498-504.

14. Hangarter, R., S. K. Ries and P. Carlson. 1978. Effect of triacontanol on plant cell cultures in vitro. Plant Physiol. 61:855-857.

15. Hawker, J. S., H. Marschner and A. Krauss. 1979. Starch synthesis in developing potato tubers. Physiol. Plant 46:25-30.

16. Henry, E. W. and D. J. Prino. 1979. The effects of triacontanol on seedling growth and polyphenol oxidase activity in dark and light grown lettuce. J. Plant Nutr. 1(4):397-405.

17. Hoagland, R. E. 1980. Effects of triacontanol on seed germination and early growth. Bot. Gaz. 141(1):53-55.

18. Hodge, J. E. and B. T. Hortreike. 1962. Determination of reducing sugars and carbohydrates Phenol-sulfuric acid method. In R. L. Wistler, M. L. Wolfram, eds., Methods of Carbohydrate Chemistry. I. Analysis and preparation of sugars. Academic Press, New York, pp. 388-389.

19. Jones, J., V. Wert and S. K. Ries. 1979. Specificity of 1-triacontanol as a plant growth stimulator and inhibition of its effect by other long chain compounds. Planta 144:277-282.

20. Kar, M. and D. Mishra. 1976. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol. 57:315-319.

21. Khanna, S. K., P. S. Krishnan and G. G. Sanwal. 1971. Inhibition of glucan phosphorylase in the leaves of Dendrophthoe falcata. Phytochemistry 10:545-550.

22. Khanna, S. K., P. S. Krishnan and G. G. Sanwal. 1971. Glucan phosphorylase in the leaves of Dendrophthoe falcata: purification and characterization of enzyme. Phytochemistry 10:551-559.

23. Kinoshita, K., H. Ishikawa and K. Shinoda. 1958. Solubility of alcohols in water determined by the surface tension measurements. Bul. Chem. Soc. Japan 31:1081-1982.

24. Knowles, N. R. 1980. Apparent increases in total plant nitrogen following applications of triacontanol. M. S. Thesis, Michigan State University, East Lansing, MI.

25. Kolattukudy, P. E. 1969. Plant waxes. Lipids, 5: 259-275.

26. Kolker, L. 1978. Analytical procedures for 1-triacontanol and its presence in plants and the environment. M. S. Thesis, Michigan State University, East Lansing, MI.

27. Lee, Y. P. 1960. Potato phosphorylase I. Purification, physiochemical properties and catalytic activity. Biochem. Biophys. Acta. 43:18-24.

28. Lee, Y. P. 1960. Potato phosphorylase II. Phosphate and sulfhydryl groups. Biochem. Biophys. Acta. 43: 25-30.

29. Lin, Willy. 1979. Potassium and phosphate uptake in corn roots. Plant Physiol. 63:952-955.

30. Lowe, R. H. and J. L. Hamilton. 1967. Rapid method for the determination of nitrate in plant and soil extracts. J. Agr. Food Chem. 15:359-361.

31. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193:268-275.

32. Madsen, E. 1968. Effect of CO_2-concentration on the accumulation of starch and sugar in tomato leaves. Physiol. Plant 21:168-175.

33. Marcelle, R. D. and A. Chrominski. 1978. Growth regulating activity of triacontanol. Page 116 in M. Abdel-Rahman, ed. Proceedings of the 5th Annual Meeting of Plant Growth Regulator Working Group. Plant Growth Regulator Working Group, Blacksburg, VA.

34. Marwaha, R. S. and B. O. Julino. 1976. Aspects of nitrogen metabolism in the rice seedling. Plant Physiol. 57:923-927.

35. Molotkovski, Y. G. and I. M. Zhestkova. 1966. Morphological and functional changes in isolated chloroplasts under the influence of oleate. Biochem. Biophys. Act. 112:170-172.

36. Nafzinger, Emerson D. and H. R. Killer. 1976. Influence of leaf starch concentration on CO_2 assimilation in soybean. Plant Physiol. 57:560-563.

37. Nakamura, K., A. Kuwahara and K. Takeo. 1979. Study of the interaction between phosphorylase and hydrophobic groups by means of affinity electrophoresis. J. Chromatogr. 171:89-99.

38. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

39. Okamoto, T., S. Kutoh and S. Marakami. 1977. Effects of linolenic acid on spinach chloroplast structure. Plant and Cell Physiol. 18:551-560.

40. Peterson, L. A. and G. Chesters. 1964. A reliable total nitrogen determination on plant tissue accumulating nitrate nitrogen. Agronomy Journal 56:89-90.

41. Pridham, J. B. 1965. Low molecular weight phenols in higher plants. Ann. Rev. Plant Physiol. 16:13-36.

42. Ries, S. K., H. Bittenbender, R. Hangarter, L. Kolker, G. Morris and V. Wert. 1977. Improved growth and yield of crops from organic supplements. Agriculture and Energy, Academic Press Inc., New York, pp. 377-384.

43. Ries, S. K., T. L. Richman and V. F. Wert. 1978. Growth and yield of crops treated with triacontanol. J. Amer. Soc. Hort. Sci. 103(3):361-364.

44. Ries, S. K. and V. Wert. 1977. Growth response of rice seedlings to triacontanol in light and dark. Planta 135:77-82.

45. Ries, S. K., V. Wert, C. C. Sweeley and R. A. Leavitt. 1977. Triacontanol: A new naturally occurring plant growth regulator. Science 195:1339-1341.

46. Rosen, H. 1956. A modified ninhydrin colorimetric analysis for amino acids. Archives Biochem. and Biophy. 67:10-15.

47. Sagaral, E. G., D. M. Orcutt, and C. L. Foy. 1978. Influence of time and rate of triacontanol applications on the growth and yields of selected plants. Page 115 in M. Abdel-Rahman, ed. Proceedings of the 5th Annual Meeting of Plant Growth Regulator Working Group. Plant Growth Regulator Working Group, Blacksburg, VA.

48. Siegenthaler, P. A. 1973. Change in pH dependence and sequential inhibition of photosynthetic activity in chloroplast by unsaturated fatty acids. Biochem. Biophys. Acta. 305:153-162.

49. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. 195:19-23.

50. Steffens, G. L., T. C. Tso and D. W. Spaulding. 1967. Fatty alcohol inhibition of tobacco axillary and terminal bud growth. J. Agric. Food Chem. 15:972-975.

51. Stowe, B. 1958. Growth promotion in pea eipcotyl sections by fatty acid esters. Science 128:421-423.

52. Stowe, B. and M. A. Dotts. 1971. Probing a membrane matrix regulating hormone action. Plant Physiol. 48:559-565.

53. Taussky, H. H. and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202:675-679.

54. Tulloch, A. P. and L. L. Hoffman. 1974. Eipcuticular waxes of Secale cereale and Triticale hexaploide leaves.

55. Turner, J. F. and D. H. Turner. 1975. The regulation of carbohydrate metabolism. Ann. Rev. Plant Physiol. 26:159-186.

56. Vlitos, A. J. and D. G. Crosby. 1959. Isolation of fatty alcohols with plant-growth promoting activity from Maryland Mammoth tobacco. Nature 184:462-463.

57. Warth, A. H. 1956. The chemistry and technology of waxes, 2nd ed. 948 pp. Reinhold Publishing Corp., New York, N.Y.

58. Yamaya, T. and K. Ohira. 1978. Nitrate reductase inactivating factor from rice seedlings. Plant and Cell Physiol. 19(2):211-220