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APPARENT INCREASES IN TOTAL PLANT NITROGEN
FOLLOWING APPLICATIONS OF TRIACONTANOL

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APPARENT INCREASES IN TOTAL PLANT NITROGEN FOLLOWING APPLICATIONS OF TRIACONTANOL

by

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ABSTRACT

APPARENT INCREASES IN TOTAL PLANT NITROGEN

FOLLOWING APPLICATIONS OF TRIACONTANOL

by

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Triacontanol stimulates an increase in growth and apparent Kjeldahl nitrogen (N) of rice seedlings (Oryza sativa L.) and N in supernatants of rice and corn (Zea mays L.) within 40 minutes. The in vitro N response is dependent upon atmospheric N which does not serve as substrate for the increase. Atmospheric substitution and $^{15}N_2$ enrichment experiments established that the apparent N increase in rice seedlings was independent of atmospheric N. TRIA increased the soluble N pools of the plant, specifically the free amino acid and soluble protein fractions. No differences in depletion or enrichment of ^{15}N incorporated into soluble and insoluble N fractions of rice could be detected. The apparent total N increases, therefore, appear to be an artifact of Kjeldahl analysis resulting from a TRIA stimulated change in the chemical composition of the seedlings.

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INTRODUCTION

Evidence of the growth-promoting effects of long chain alcohols can be traced back to 1959 when Crosby and Vlitos (6) demonstrated growth enhancement in Avena coleoptiles. Recent work with triacontanol (TRIA), a naturally occurring, 30-carbon, aliphatic, primary alcohol [$CH_3(CH_2)_{28}CH_2OH$], has shown that it stimulates an apparent total N increase when applied to rice and corn at concentrations ranging from 1 to 1000 μg/L (36). Total Kjeldahl nitrogen (N) increases have been found to be linear with time, over short time intervals, and can be measured within 10 min in corn seedlings and 40 min in rice seedlings (Ries, unpublished data). Hangarter et al., (17) demonstrated that TRIA significantly increases the protein content of haploid tabacco cell cultures. The response was dependent upon light and involved an increase in cell number indicating a stimulatory effect on the rate of protein synthesis.

This study was initiated in an attempt to reveal the source, fate and distribution of the reduced N responsible for the rapid increase in Kjeldahl N in vivo, in TRIA treated rice seedlings, and in vitro in corn extracts. The source of the N was investigated by nitrate analysis, nitrate reductase assays and atmospheric substitution experiments. Mass spectrometric techniques, utilizing an isotope ratio mass spectrometer, have been employed to detect the movement of heavy N (^{15}N) into, as

well as within, the plant. Relative levels of ^{15}N in soluble and insoluble N pools of treated and control plants were compared.

LITERATURE REVIEW

Current increases in energy costs, coupled with an ever expanding world population, necessitates more efficient production of important food crops. The expenses involved in production and application of N fertilizers remain the single most critical input for obtaining higher crop yields (12). These costs can be kept minimal and yields could be be increased per unit area to help meet present demands by increasing the efficiency of N utilization by plants. This discussion briefly summarizes two approaches to achieving these goals: genetic selection and growth regulation by chemicals. The pertinent literature on TRIA as a possible stimulator of N metabolism in crops is also reviewed.

Genetic Selection

Genetic enhancement of N acquisition and assimilation by plants can be achieved through breeding for high protein content and increased nitrate uptake and reduction. In rice, nitrate absorbed by the roots is translocated to the leaves where it is reduced to ammonia and incorporated into α keto acids by reductive amination (30). Similarly, high levels of nitrate reductase (NR) have been reported for the leaf tissue of corn indicating that this is the primary location of nitrate assimilation (41).

Attempts have been made to utilize levels of the enzyme NR as a criterion for genetically selecting high grain protein varieties. Early

studies on corn revealed direct relationships between soluble leaf protein and NR activity. However, grain yields showed no such relation (15,45). In wheat, positive correlations between NR activity and grain N are only evident when sufficient supplies of nitrate are available to the plant (7,11). Deckard et al., (8) explained that since nitrate uptake shows diurnal fluctuations, high NR genotypes would not be expressed under low nitrate regimes, due to the fact that their outputs of mid-day reduced N would be significantly less than indicated in enzyme assays.

Recent work has revealed that genotypes differing in nitrate absorption, nitrate content and NR activity do not always display varying protein levels (22). Other physiological parameters such as amounts of protein reserves and rates of translocation of N from vegetation to the maturing grain play important roles in determining final protein content and must be considered in screening for high protein genotypes (22).

Growth Regulation by Chemicals

Stimulation of N utilization by chemical means has been an area of relatively intense research the past two decades. Wray et al., (43) proposed that the efficiency of protein accumulation could be enhanced by hastening synthesis or slowing natural breakdown processes. The use of growth-regulating chemicals to accomplish this might result in more efficient N utilization, higher protein content and possibly improved protein quality. Ultimately, an increase in grain protein would be achieved, perhaps resulting in enhancement of the nutritional status of populations dependent upon cereals as major protein sources (22).

In environments where nitrate is not limiting, the rate of assimilation of N into reduced organic forms is largely dependent on the

activities of nitrate and nitrite reductase. Nitrate reductase, a substrate inducible enzyme, has been found to be stimulated by sub-lethal doses of several herbicides. Ries et al., (34) reported that simazine, a triazine herbicide, significantly accelerated the rate of protein accumulation in rye and peas. It was confirmed that, at least in rye and oats, simazine exerted its effect by stimulating NR activity and the increased protein which resulted contained no new protein types.

Enhanced NR activity in maize and cucumber (Cucumis sativus L.) following treatment with 2,4-dichloro-phenoxyacetic acid (2,4-D) has been reported by Beevers et al., (1). Sub-lethal doses of 2,4-D have been found to significantly increase yields of sugar beet (Beta vulgaris L.), potatoes (Solanum tuberosum L.) and other crops in field trials (42). A summary of the published data indicates that 2,4-D influences the distribution of plant protein while increasing the over-all total N content, possibly through enhanced NR activity (29).

Total N content of plants can also be increased by non-herbicidal compounds. In 1975, it was shown by Ries et al., (35) that coarsely ground alfalfa hay (Medicago sativa L.) significantly increased the yield and total N content of several commercially important vegetable species. Realization of the fact that the observed increases with alfalfa were greater than those achieved with equivalent amounts of fertilizer led to an extensive search for the responsible compound(s). Isolation of the growth-promotor in crystalline form was achieved from a chloroform extract of alfalfa meal. It was identified as 1-triacontanol via mass spectrometry (33).

TRIA was first identified in alfalfa by Chibnall (5) and was later shown to be a constituent of the cuticular waxes of many plant species

(26). The mechanism by which this compound influences plant growth remains obscure, however, the magnitude of the levels needed to produce a response (1 to 1000 μg/L) suggest a hormone-like activity.

Jones et al., (24) demonstrated that a chain length of 30 carbons coupled with a terminal hydroxyl group was specific for TRIA's growth-enhancing activity. Tests of the growth-promoting activity of TRIA analogs varying in chain length from 16 to 32 carbons proved negative. In fact, these compounds resulted in inhibition of the TRIA response when applied simultaneously (24). Gross (14) has shown that several aliphatic alcohols with chain lengths from 9 to 11 carbons significantly inhibit bud growth.

Recent tests on 15 weed, crop and horticultural species have shown no effect of TRIA on germination and early growth, however, an inhibition of axis elongation was apparent in three of the test species (20). TRIA was shown to enhance growth of Great Lakes lettuce (Lactuca sativa L.) roots in both light and dark grown seedlings (19). Enhanced polyphenol oxidase activity following TRIA application has been demonstrated in lettuce leaf tissue (19).

Both TRIA isolated from alfalfa, and synthetic TRIA (Analabs, North Haven, Conn.), will stimulate increases in dry weight, leaf area and N content when applied to rice and corn at concentrations ranging from 1 to 1000 μg/L (36). In rice, the response proved to be independent of light conditions and CO_2 concentration appeared to play a regulatory rather than a substrate role (3). This was supported by the fact that TRIA induced dark responses could be eliminated by removing atmospheric CO_2 (36). Six h dark responses were characterized by increases in dry weight, soluble and insoluble Kjeldahl N and soluble carbohydrates.

Metabolic profiling results, utilizing rice grown in nutrient media containing deuterium oxide and harvested at intervals after treatment, showed that TRIA somehow increases the incorporation of carbon into most tricarboxylic acid intermediates (9). Both carbon and N incorporation into the α-amino acids were also found to increase over control plants within 10 min. It has been hypothesized that TRIA either increases the mobilization of N from the roots or somehow increases amino acid pools making them available for increased protein synthesis (9).

MATERIALS AND METHODS

Preparation of Plant Materials

Rice seed, 'IR-8' or 'ESD 7-1' (Calif.), was surface sterilized with a 0.1% (w/v) solution of mercuric chloride. The seeds were planted in 77 ml plastic cups containing turface (Wyandatte Chemical Company, Detroit, MI) and watered with distilled water to the point of saturation. Growth conditions were maintained on a 16 h photoperiod at 30°C and an 8 h night at 25°C with 7.0 $\mu W/cm^2$ in the phytosynthetically active region as measured from the top of the canopy. At the 7 to 11-day-old stage the seedlings were transplanted into 220 ml plastic cups containing 180 ml of quarter-strength Hoagland's solution (pH 4.5) having 3 mM nitrate N (21). Four seedlings were suspended in the solution by a foam rubber disc in the top of the cup. The cups were wrapped in aluminum foil to exclude light. Nutrient solutions were renewed every 2 to 3 days thereafter with half-strength Hoagland's containing 6 mM nitrate N.

Field corn, 'Pioneer 3780', was sown in 18 cm clay pots (8 seeds/pot) containing a sterilized soil mix of equal volumes peat, sand and sandy loam soils. The pots were placed in the greenhouse with the night temperature maintained at 25°C and a day temperature averaging 30°C. At the 7-day-old stage a fertilization program was initiated with the seedlings receiving a soluble 20-20-20 fertilizer twice a week at a concentration of 1 g/L. The N in the fertilizer was composed of 5.61% nitrate, 3.96% ammonia and 10.43% urea. All plant materials received fertilizer

or fresh nutrient solution the night before an experiment took place.

Preparation of Treatment Solutions

Treatment solutions for the NR experiments were prepared from stocks of pure TRIA (American Cyanamid, Princeton, NJ) dissolved in chloroform and added directly to the infiltration media (see below). Solutions for all other experiments were prepared from stocks of 0.1 to 1.0 mg/g TRIA-Tween 20 (polyoxyethylene sorbitan monolaurate) emulsions. The amount of stock added to glass distilled water was adjusted to achieve a final concentration of 0.1% (w/v) Tween 20 and 100 to 1000 μg/L TRIA.

Nitrate and Nitrate Reductase Analysis

The NR activity of corn leaf tissue was measured by both an *in vivo* (16) and an *in vitro* (39) colorimetric assay. The methods of Lowe and Hamilton (28) were employed to analyze free nitrate in rice tissues.

For the *in vivo* experiments, freshly harvested corn leaves were cut into segments (2 to 5 mm) or discs (No. 3 cork borer) and placed into 50 ml Erlenmeyer flasks containing cold (3°C) infiltration media and TRIA (10 to 100 μg/L). The infiltration media consisted of 300 mM KNO_3 and 1 mM potassium phosphate (pH 7.5). The flasks were stoppered and repeatedly evacuated (35 mm-Hg for 30 s) until the segments were visibly wetted. Aliquots of 0.1 or 0.2 ml were removed at timed intervals for determination of nitrite. In all tests, variation due to differences in fresh weight and positions on the leaf from which the segments were taken, was accounted for using a randomized complete block design.

For the in vitro experiment, corn was germinated in 18 cm clay pots containing soil in the greenhouse and transferred to pots containing vermiculite at the 3-day-old stage. Two days later, the seedlings were suspended in 220 ml plastic cups containing 180 ml of full-strength Hoagland's solution with a double concentration of minor elements. The cups were placed in a growth chamber with continuous aeration under conditions previously described. The seedlings were treated at the 7-day-old stage by dipping in a 1000 μg/L TRIA, 0.1% (w/v) Tween 20 solution. Nitrate reductase activity was measured 3 h after treatment with the reduced NADH methods of Sanderson and Cocking (39).

Growth Response Studies: Plant Analysis

Rice seedlings were sorted and blocked for size. Six blocks were utilized in a randomized complete block design with three randomly assigned treatments. The treatments consisted of a zero-time harvest, i.e. seedlings were harvested at the outset of the experiment, a control in which the seedlings were inverted and dipped in 100 ml of a 0.1% (w/v) Tween 20 solution and a TRIA treatment in which the seedlings were dipped in a similar solution containing 100 μg/L TRIA. The treatment solutions were changed after each replicate and the roots of each seedlings were rinsed three times in distilled water before being placed in 25 mm x 200 mm, open test tubes (two seedlings/tube). The tubes contained 10 ml of half-strength Hoagland's (pH 4.5) with 1 mM N as $(NH_4)_2SO_4$. Plants were placed in a growth chamber at 30°C for 40 min after which they were harvested by separating roots from shoots, weighing the shoots, and immediately digesting the shoots for automated Kjeldahl analysis (13). Fresh weight and N content were analyzed.

Growth Response Studies: System Analysis

The system analysis experiments entailed a simple procedure in which the entire plant culture system, (nutrient solution and seedling), was digested for automated Kjeldahl analysis. Individual seedlings were cultured, sorted, treated and placed in a growth chamber for 40 min as described previously. Harvesting involved removing the plants from the growth chamber and freezing them in a dry-ice and acetone bath. The nutrient solutions, containing 1 mM N as $(NH_4)_2SO_4$, were frozen in a similar manner and lyophilized along with the seedlings. The freeze-dried plants were weighed and combined with the nutrient solutions in which they grew for estimation of total N. Treatment solutions were prepared by dissolving TRIA or tetracosanol plus TRIA in chloroform (10 mg/ml) and adding determined amounts to distilled water containing 0.1% (w/v) Tween 20 to achieve a final concentration of 100 µg/L of each alcohol.

In Vitro Methods

The in vitro methods described in this thesis were developed by Robert L. Houtz (Department of Horticulture, Michigan State University). For corn, the youngest one to three leaves were treated with a 1000 µg/L TRIA solution and ground in a cold mortar and pestle (4°C) with 4 ml grinding media for each g fresh weight of tissue. The grinding media consisted of 20 mM potassium phosphate (pH 7.2) containing 70 µL/L β-mercaptoethanol. Rice was treated with a 100 µg/L TRIA solution and ground in 5 ml grinding media for every 2 g of leaf tissue. The resulting crude homogenates were squeezed through 4 layers of cheese cloth and centrifuged for 20 min at 8000 g for corn or 5000 g for rice. The

supernatant solution was added to cold incubation media (1 ml for every 2 ml of media) and kept on ice until the initiation of an experiment (Figure 1). The incubation media was prepared by combining the following solutions shortly before the leaves were to be extracted; NADPH (Na salt) 50 mg/60 ml, NADH (Na salt) 42 mg/60 ml, ATP (Na salt) 44 mg/80 ml, $MgCl_2 \cdot 6\ H_2O$ 25 mg/125 ml, oxaloacetic acid 10 mg/60 ml and α-ketoglutarate 10 mg/60 ml. Each component solution was prepared in grinding media. An experiment began by pipetting predetermined volumes of the extract into 25 mm x 200 mm test tubes or 50 to 125 ml Erlenmeyer flasks which served as replicates. At the start of a test, a 3 ml sample was taken for N analysis from each replicate and the tubes were stoppered and placed in a water bath shaker with gentle shaking at 25 to 30°C. Similar samples were harvested from the replicates at 60, 80 or 120 min incubation. Samples were freeze-dried or digested directly for N analysis. All experiments were analyzed utilizing a completely randomized design.

Analysis of Total Nitrogen

Total N was measured utilizing the automated Kjeldahl procedure of Ferrari (13). Digestion of plant samples was accomplished by addition of a sulfuric-perchloric acid, selenium dioxide mixture composed of 40 ml perchloric acid, 1800 ml sulfuric acid and 6 g SeO_2 made up to 2 L with distilled water (4 ml acid for every 20 mg dry wt of rice). The samples were solubilized by heating until the solutions cleared. After cooling, the samples were diluted with distilled water (6 ml water for every 4 ml of acid) and poured into plastic cups which were placed in a sampling tray. The tray was attached to an Auto-Analyzer

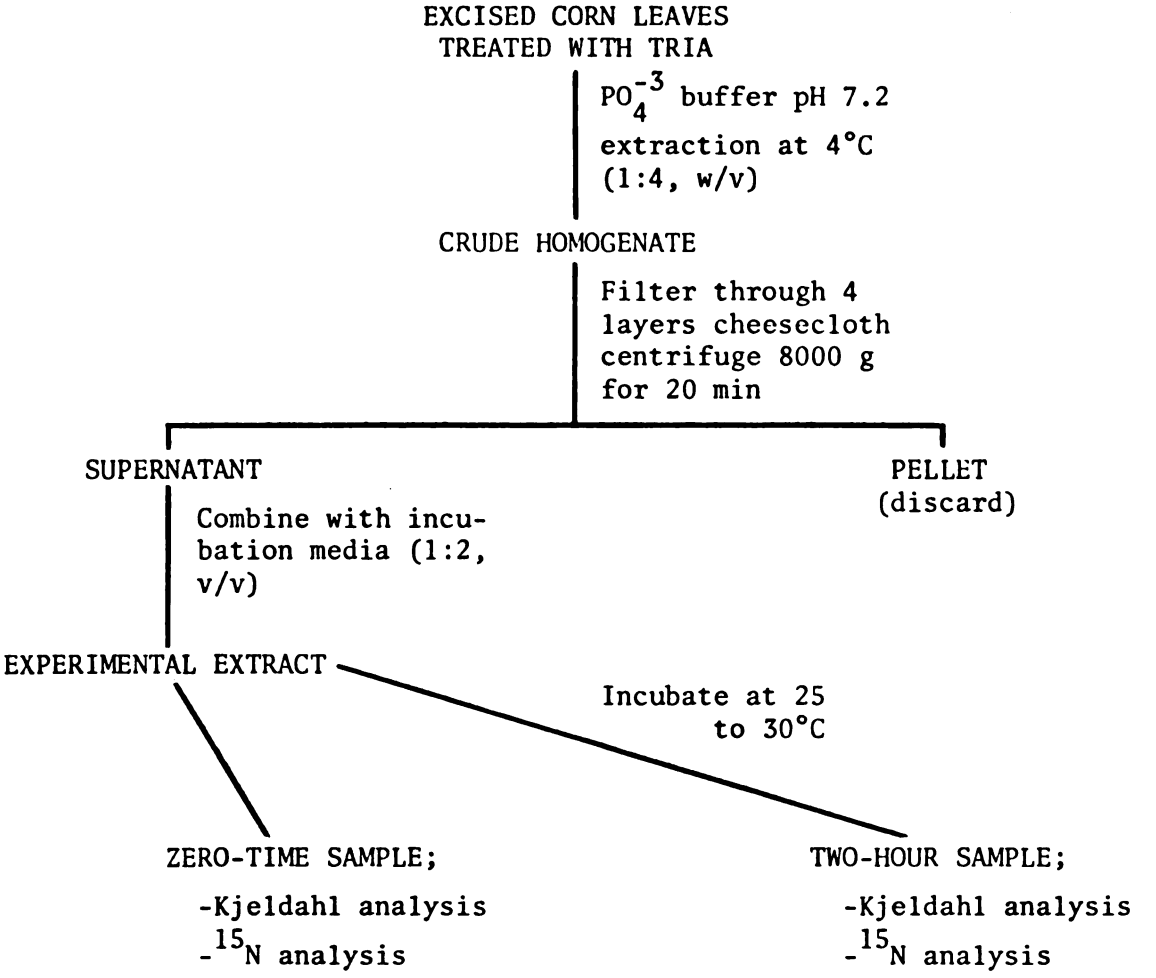


Figure 1. In vitro methods for assaying the N content of TRIA treated corn extracts over time.

(Technicon Instruments Corporation, Tarrytown, NY) for automated Kjeldahl analysis. Nitrogen was detected by an alkaline-phenol color reaction with maximal absorbance at 632 nm. Standards, consisting of 10 to 50 mg of ground wheat (4.34% moisture, 2.566% N) were analyzed with each experiment for calibration purposes. Percentage N in the ground wheat was established utilizing orchard leaves obtained from the National Bureau of Standards, Washington, D.C. (standard reference material 1571). Modifications of the procedure involving addition of salicylic acid to include estimates of nitrate N and other high valence forms were not employed (31). Hence, total N refers to the total Kjeldahl detectable N utilizing the above procedures.

Atmospheric Substitution Procedures

For the in vitro atmosphere experiments, N_2, argon, oxygen and carbon dioxide were mixed with the aid of a proportioner and admitted to the experimental flasks through serum stoppers. The gasses were proportioned to achieve an 80% argon or N_2 atmosphere while O_2 and CO_2 remained at their normal levels (20% O_2, 0.033% CO_2). The gas mixtures were admitted at a flow rate of 12 ml/s, for 30 s, through a disposable syringe needle puncturing the serum bottle stopper. A vent needle was also utilized during the gassing procedure to allow flushing of the flasks. Similar methods were used to change the atmosphere around excised rice shoots which were treated as previously described. The shoots were placed in 35 mm x 300 mm test tubes and gassed for 30 s through serum bottle stoppers at a flow rate of 16 ml/s.

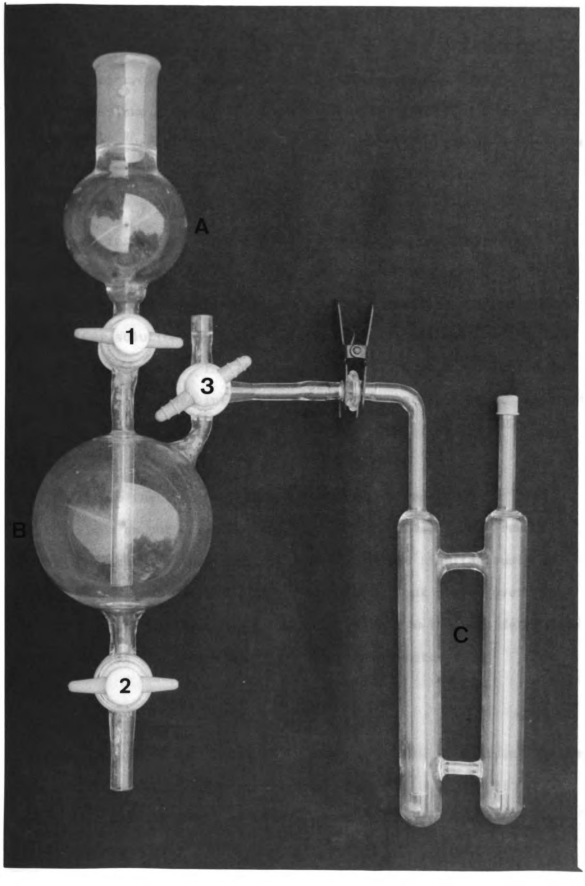
Acetylene was generated by the Kipp reaction which involves the addition of water to calcium carbide pellets. The resulting acetylene

was stored over a saturated solution of Na_2SO_4. In the acetylene test for nitrogenase activity (18), in vitro rice extracts added to 35 ml gas bottles were sealed with serum bottle stoppers. The bottles were flushed with an 80% Ar, 20% O_2 and 0.033% CO_2 mixture for 30 s at a flow rate of 17 ml/s. Three ml of acetylene was injected into the appropriate treatments by first withdrawing 3 ml from the bottles and then injecting the acetylene. This created approximately a 13% acetylene atmosphere in each bottle. After taking 1 ml, zero-time gas samples, the bottles were placed in a water-bath shaker with gentle shaking at 28°C. Final gas samples were analyzed at the end of a 70 min incubation period and compared with the zero-time samples. Analysis of ethylene in all samples was accomplished utilizing a Varian model 3700 gas chromatograph with a 2 m Porapak-N column. Optimal sensitivity was achieved with an injection temperature of 120°C and an N_2 flow rate of 30 ml/min.

Generation of $^{15}N_2$

A N gas generating apparatus was constructed as modified from Rittenburg (37) by David R. Dilley (Professor of Horticulture, Michigan State University) (Figure 2). Ammonium sulfate containing a 99.9% ^{15}N label was obtained from Monsanto Research Corporation (Mound Laboratory, Miamisburg, OH) and 1.754 g was added to reservoir A with stopcock 1 in the closed position (Figure 2). A minimal amount of water was admitted for complete dissolution of the $(NH_4)_2SO_4$ and 2 or 3 drops of 5% (w/v) H_2SO_4 was added for acidification. With stopcock 2 in the closed position, reservoir B and gas trop C were evacuated (30 mm-Hg) by opening the three-way stopcock (3) which was then

Figure 2. A N gas generating apparatus as modified from Rittenburg (37).


A
1
3
B
C
2

closed to maintain the vacuum in the apparatus. The $(NH_4)_2SO_4$ solution in reservoir A was de-gassed of N_2 with helium or argon and slowly admitted to reservoir B. Approximately 1.5 ml of de-gassed water was used to rinse the sides of A and was similarly admitted to B preserving as much of the vacuum in the apparatus as possible. Lithium hypobromite solution, prepared by adding 2 ml of reagent grade bromine to 60 ml of a cold (0 to 5°C) 10% (w/v) solution of lithium hydroxide, was slowly admitted to reservoir B until the reaction had visibly stopped and no more could be added. At this point, gas trap C was immersed in liquid N_2 which created a negative pressure in the apparatus. The remainder of the LiOBr solution was added (approximately 75 ml) until all of the $(NH_4)_2SO_4$ had reacted. The liquid N_2 was removed and the solution in reservoir B was drained into a waste container via stopcock 2. Reservoir A was filled with a saturated Na_2SO_4 solution for displacing $^{15}N_2$ from the apparatus.

To sample the $^{15}N_2$, the liquid N was placed under the gas trap and a maximum amount of Na_2SO_4 was added to B. The gas was drawn through the septum with a disposable syringe while simultaneously admitting an equal volume of Na_2SO_4. Labelled gas was stored over a saturated Na_2SO_4 solution and found to be 98.98 atom % ^{15}N as determined on a Varian MAT GD-150 isotope ratio mass spectrometer.

Atmosphere Enrichment

The atmosphere enrichment experiments consisted of exposing treated rice seedlings and *in vitro* corn extracts to various levels of $^{15}N_2$. Plants were treated with test solution and placed in 35 mm x 300 mm test tubes, (or 250 ml Erlenmeyer flasks for the *in vitro* extracts),

containing half-strength Hoagland's solution (pH 4.5) with 1 mM N as $(NH_4)_2SO_4$. The amount of Hoagland's solution was adjusted so that the available gas space within each tube was 154 ml. The tubes were closed with rubber stoppers, bored to accommodate septums, through which gas samples could be exchanged. Atmosphere enrichment was accomplished by replacing 10 to 25 ml of the atmosphere surrounding the plant or extract with an equivalent volume of $^{15}N_2$. Plants were harvested after 40 min incubation by freezing in dry-ice and acetone and lyophilizing. Dried plant material was ground in a Wiley mill (40 mesh screen) with aliquots taken for total N analysis and ^{15}N analysis utilizing a VG Micromass MM-622 isotope ratio mass spectrometer.

The experimental sequence of the in vitro experiment appears in Figure 1. Three replicates were used in a completely randomized design. Heavy N gas was admitted to the flasks containing the experimental extracts after taking the zero-time samples. Samples were taken for both total N and mass spectrometric (MS) analysis. Total N samples were digested immediately and samples for MS analysis were dried at 72°C in preparation for micro-Kjeldahl digestion.

Preparation of Samples for MS Analysis

The micro-Kjeldahl procedures of Black (4) were employed for the digestion and distillation of plant samples for MS analysis. Catalyst, consisting of a 1:10:100 mixture of SeO_2, $CuSO_4$ and K_2SO_4, was added to previously ground plant material (1 g catalyst for each mg total N in sample). Sulfuric acid was added to the plant-catalyst mixture (3 ml for each mg total N) and the flasks were placed on burners for digestion. Samples were digested for approximately 6 h during which time the organic N was converted to $(NH_4)_2SO_4$.

The NH_4^+ was liberated by steam distillation following addition of 10 N NaOH to the digestion mixture. Eighty percent ethanol was run through the distillation apparatus between samples to prevent cross contamination. The resulting free NH_4^+ was trapped in 10 ml of 0.1 N HCl and dried at 72°C resulting in pure NH_4Cl crystals. The MS analysis of ^{15}N was accomplished by the addition of LiOBr solution to the crystals and admitting the resultant gas into the mass spectrometer. All samples for MS analysis contained from 2 to 4 mg total N.

Depletion and Distribution Analysis

Nitrogen-15 depletion experiments involved culturing rice seedlings on $K^{15}NO_3$ (5.3 atom %) for 20 to 96 h prior to treatment with TRIA. Plants were sorted for size and suspended in 220 ml plastic cups containing 180 ml of half-strength Hoagland's solution with 6 mM $K^{15}NO_3$ as the sole source of N. For the 20 h experiments, 12 to 16 cups, each containing four to six seedlings of uniform size, were placed in a growth chamber for the duration of the prelabelling period. Following the 20 h interval, the seedlings in each cup were sorted again, randomized and treated by dipping in the appropriate test solution. Treated plants were placed in 25 mm x 200 mm test tubes containing 6 to 10 ml of labelled or unlabelled Hoagland's solution. After 80 min incubation in a growth chamber, the plants were harvested by separating roots from shoots, freezing both in dry-ice and acetone, and lyophilizing or oven drying at 72°C.

Seedlings in blocks I through IV, V through VIII and IX through XII were combined yielding a total of three blocks and three treatments with four plants/treatment. This was necessary in order to assure

enough plant material for fractionation procedures. The dried shoots were ground in a Wiley mill. Aliquots were taken for total N, soluble protein, free amino acid, nitrate and ^{15}N analysis. In most experiments the shoot material alone was analyzed. However, in the soluble-insoluble fractionation experiment 20 mg aliquots of the shoot material were combined with proportionate amounts of root material as determined on the basis of the original shoot to root dry weight ratio.

The 96 h experiment necessitated the addition of labelled Hoagland's solution to the cups twice over the 4-day interval. Six blocks and three treatments (four plants/treatment) were utilized in a randomized complete block design. Seedlings were treated and incubated in 220 ml cups containing unlabelled Hoagland's solution for 80 min. Plants were harvested by freezing and lyophilizing.

For the in vitro depletion experiment, 73 mg of $K^{15}NO_3$ (5.3 atom %) was added to 158 ml of incubation media which was combined with supernatant solution of corn leaf extracts yielding replicates (Figure 1). Zero-time aliquots for total N and ^{15}N analysis were sampled and the extracts were placed in a water-bath shaker (25 to 30°C) for the duration of the 120 min incubation period. All samples were dried at 72°C prior to N analysis.

Fractionation Procedures

The N distribution experiments involved separation of the plant material into various N fractions followed by ^{15}N analysis of each fraction. The 17 to 19-day-old rice seedlings were prelabelled and treated as discussed in the previous section. An aliquot of the dried plant material, containing 2 to 4 mg of water soluble N, was extracted

in a mortar and pestle with glass distilled water (1 ml for every 10 mg of plant material). The resulting extract was centrifuged at 10,000 g for 30 min and the supernatant solution was set aside. The pellet was washed with an equivalent volume of water and recentrifuged. Both supernatants were recentrifuged (10,000 g for 30 min) and the pellets were combined by transferring to 125 ml Erlenmeyer flasks with 25 ml of distilled water. The resulting supernatant solutions were also transferred to 125 ml flasks and all fractions were dried at 72°C. Separation of the plant material into water soluble and water insoluble N fractions was thus achieved.

Lyophilized plant material was utilized in experiments involving MS analysis of soluble and insoluble N fractions. Separation into soluble and insoluble portions was achieved as discussed previously and the soluble protein was precipitated with 20% trichloroacetic acid (TCA). Isolation of the protein was accomplished by centrifugation (10,000 g for 30 min) and the resulting protein pellet was transferred to 125 ml Erlenmeyers with 20% TCA and dried at 72°C.

Quantitative determinations of soluble protein N, free amino N and nitrate N were made by either assaying the supernatant solutions in the regular fractionation sequence or utilizing aliquots of the dried plant material specifically for this purpose. Soluble protein N was determined by a modification of the Lowry procedure (2). The methods of Hyman Rosen (23) were used in assaying free amino N levels.

Statistical Procedures

In all rice seedling experiments, a randomized complete block design was utilized to remove variance due to differences in plant

size. Prior to the initiation of a test, the seedlings in each block were assigned to a particular treatment utilizing a random number table. In most tests, six blocks and three treatments were employed and coefficients of variation ranged from 1 to 6%. Orthoganol comparisons were utilized in separating treatment effects from controls. F tests and LSD's were used to compare means where appropriate.

Analysis of variance for the in vitro experiments was accomplished using a completely randomized design. Replicates were formed by pipetting aliquots of extract into labelled Erlenmeyer flasks containing incubation media. Samples were harvested at intervals for analysis of N. Since the extract had been previously treated with TRIA, the effect of time on N content was being tested, i.e. time served as treatment. Coefficients of variation in these tests ranged from 1 to 4%. Where appropriate, orthoganol comparisons were utilized.

RESULTS AND DISCUSSION

Nitrate Reductase Experiments

It was hypothesized, that the apparent total N increase in both corn and rice, was caused by a TRIA induced stimulation of NR activity. This would result in enhanced reduction of nitrate within the leaf tissue and ultimately a larger pool of total N would be available for detection by Kjeldahl. In rice (44), measurements were restricted to endogenous nitrate levels which indirectly revealed the role of NR in the TRIA response. These results will become apparent in later sections. Direct measurements of NR activity in corn was accomplished by employing both *in vivo* and *in vitro* procedures.

The results of a corn leaf NR experiment, utilizing the *in vivo* assay methods, appear in Table 1. No significant effect on NR could be demonstrated over the times or concentrations of TRIA employed.

Table 1. Effect of increasing concentration of TRIA on nitrite accumulation from corn leaves measured at various times after treatment with the *in vivo* assay.

TRIA (mg/L)	Nitrite Accumulation ($m\mu mol\ NO_2^-/h/g$ dry wt) 30 min	60 min	120 min
0.00	4,470	11,042	15,226
0.01	3,050	11,827	15,557
0.05	3,482	11,077	14,663
0.10	3,400	8,878	11,937

Other *in vivo* tests were conducted utilizing similar incubation times and TRIA concentrations with results showing no effects on enzyme activity due to TRIA.

To further substantiate these results, an experiment employing the *in vitro* methods of NR analysis was conducted. Corn seedlings, grown in nutrient culture, were treated at the 7-day-old stage and NR activity was measured 3 h after treatment. Once again, no significant effect on NR activity was achieved with the TRIA treatment (Table 2).

Table 2. Effect of TRIA on NR activity in corn leaves measured 3 h after treatment with the *in vitro* assay.

TRIA (0.1 mg/L)	NR Activity (mμmol NO_2^-/h/g fresh wt)
-	6,093
+	5,919

Many more tests, utilizing both assay methods, were conducted with the results supporting the null hypothesis. The conclusion that TRIA does not enhance the activity of corn NR was, therefore, accepted.

Growth Response Studies: Plant Analysis

Attention was turned toward experiments designed to characterize the total N increases at the whole plant level. A 40 min experiment comparing the fresh weight and N content of control and treated rice shoots indicated that the TRIA treated seedlings significantly gained in fresh weight and total N when compared with controls (Table 3). The apparent N increase paralleled the increase in fresh weight with no change in N concentration evident. Hence, the total N increase was due to the larger plants resulting from TRIA treatment.

Table 3. Growth response and total N content of TRIA treated rice seedlings.

Time (min)	TRIA (100 μg/L)	Fresh wt (mg/shoot)	Total N (mg/shoot)
0	0	215	1.98
40	-	223	1.97
40	+	252**	2.20**

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

A definitive explanation of the data was complicated by the fact that the increase in total N was larger than the amount of N provided to the plant through the nutrient solution (10 ml of 1 mM N as $(NH_4)_2SO_4$). It thus appeared that treating the seedlings with TRIA enabled them to acquire N other than that directly provided via the nutrient culture.

Growth Response Studies: System Analysis

In an effort to further define the role of the nutrient solution in the N increase, experiments were conducted where the total N in both plant and nutrient culture was analyzed. The data was expressed as total system N (mg/system). After 40 min incubation, a significant 21 and 24 percent increase in total system and total plant N occurred, respectively with the TRIA treatment (Table 4). An increase in concentration of N was evident from the significant rise in system N. Once again, the increase was too large to be attributed to N provided to the plants through the nutrient solution.

Table 4. Total N content of TRIA treated rice seedlings.

Time (min)	TRIA (100 μg/L)	Total N (mg/system)	(mg/plant)
0	0	1.69	1.53
40	-	1.73	1.57
40	+	2.10**	1.94**

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

A similar experiment was conducted, however, a treatment of TRIA combined with tetracosanol was added. Single rice seedlings were exposed to foliar applications of the appropriate test solution and incubated in a growth chamber for 40 min prior to system analysis. As discussed previously, tetracosanol has been shown to be an effective inhibitor of TRIA (24). No significant change in dry weight could be detected in any of the treatments after the 40 min growth period (Table 5). The total N content of the TRIA treated plants and plant-nutrient culture systems was 12 and 7 percent higher, respectively than the other two treatments. Tetracosanol resulted in complete inhibition of the TRIA induced N accumulation effect confirming earlier research performed in this laboratory (24).

Table 5. Growth response and total N content of rice seedlings exposed to TRIA or TRIA + Tetracosanol.

Treatment (100 μg/L)	Dry wt (mg/plant)	Total N (mg/system)	(mg/g dry wt)[@]
Control	42.7	1.70	34.6
TRIA	41.2	1.83**	39.1**
TRIA + Tetracosanol	43.0	1.71	34.9

[@]mg plant N/g dry wt of plant (Hoagland's N subtracted).
**F value for comparison with controls significant at the .01 level.

Atmospheric Substitution

Since the total system N increased, it was hypothesized that the source for the rapid accumulation of N in TRIA treated plants was the atmosphere. This becomes feasible when considering the possibility that TRIA may be inducing N_2 fixation through its influence on phylloplane bacteria or blue-green algae in close association with the roots.

Estimates of the rates of N_2 fixation by phylloplane bacteria and blue-green algae are available. Bacteria in the leaves of Douglas Fir (Pseudotsuga menziensii Franco) fixed a maximum of 5.2 mg N/g leaf N over a one month period (25). Similarly, Lovett and Sagar (27) demonstrated the presence of free-living N_2 fixing bacteria in the leaves of Comelina sativa (L.) Crantz. Rates for blue-green algae have been determined by Stewart (40) as ranging from 10 to 78 kg N_2 fixed/ha for a rice crop on an annual basis. The apparent increases in total N content of plants due to TRIA are on the order of 1000 times these values. However, the above estimates resulted from research performed in natural environments. These workers did not attempt to maximize rates of fixation through optimizing growth conditions or chemical stimulation. Therefore, the decision was made to test the possibility of atmospheric N_2 being directly involved in the TRIA induced, total N accumulation effect through these types of symbiotic relationships.

Both the in vitro and whole seedling systems were utilized in testing the role of atmospheric N_2 in the TRIA response. The apparent total N content of an in vitro corn system, measured at various intervals over a 2 h period, was increased by TRIA (Table 6).

Table 6. Total N content of the supernatant from TRIA (1000 μg/L) treated corn leaves assayed as a function of time.

Time (min)	Total N (mg/g dry wt)
0	74.70**
30	81.45
60	81.80
120	82.93

**F value for comparison with treatments significant at the .01 level.

Similar N responses were observed in rice extracts (unpublished data). If the gain in total N within the extracts required the presence of atmospheric N_2, then inhibition of the response should be achievable by replacing N_2 in the air, surrounding the extract, with argon. An appropriate experiment was constructed utilizing rice extracts and the results appear in Table 7.

Table 7. Effect of atmospheric substitution on the total N content of the supernatant from TRIA (100 μg/L) treated rice leaves.

Time (min)	Atmosphere	Closed[@] System	Total N (μg/system)
0	--	0	506
60	Normal	-	504
60	Normal	+	520*
60	Ar replacing N_2	+	505
60	Total N_2	+	507

[@]Incubation tubes were either stoppered (+) or left open (-).
* F value for difference from all other treatments significant at the .05 level.

Argon appeared to inhibit the N increase characteristic of the TRIA treatment in which normal amounts of N_2, CO_2 and O_2 were supplied to

the extracts. To confirm an earlier observation, that the in vitro response was optimized when all incubation tubes were stoppered, an open-tube system was included. The tube was left open for the duration of the experiment resulting in no net N gain. The anaerobic treatment (total N_2 atmosphere) showed no gain in N. This supported the research of Bittenbender et al., (3) that CO_2 is required for the response.

In a similar in vitro rice experiment, Ar was substituted for N_2 in a normal atmosphere. A significant increase in total N occurred over the zero-time and argon treatments (Table 8).

Table 8. Influence of an argon substituted atmosphere on the total N content of the supernatant from TRIA (100 µg/L) treated rice leaves.

Time (min)	TRIA (100 µg/L)	Atmosphere	Total N (mg/system)
0	+	--	2.01
80	+	Ar replacing N_2	2.08
80	+	Normal	2.30**

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

These tests were indicative of the involvement of atmospheric N_2 in the in vitro TRIA response. Dinitrogen may be utilized indirectly via an activation role or directly through acting as substrate for fixation via nitrogenase provided through bacterial or algal contamination.

To test for the presence of nitrogenase in the in vitro rice assay a 13% acetylene atmosphere was introduced to the flasks containing the rice extracts. Gas samples were taken at zero-time and 70 min after incubation for analysis of ethylene. The rice extracts produced very little endogenous ethylene (Table 9).

Table 9. Acetylene test for the presence of nitrogenase in the supernatant of TRIA (100 μg/L) treated rice leaves.

Acetylene Atmosphere	Ethylene (μl/l) Initial	70 min
--	0.0000	0.0560
13%	0.3065	0.2837

The acetylene treatment showed no increase in C_2H_4 at the end of the incubation period. The relatively high initial C_2H_4 concentration in the zero-time sample represents C_2H_4 produced in the generation of acetylene. No measurable amount of nitrogenase existed in the in vitro rice system, therefore, the TRIA induced response may not be attributed to phylloplane bacteria. The role of rhizosphere bacteria and blue-green algae in the intact seedling assays can not be ruled out.

Atmosphere Enrichment

To test the hypothesis that the N response in intact plants directly involved atmospheric N_2, the atmosphere surrounding the seedlings was enriched with $^{15}N_2$. Analysis for enrichment of plant material was accomplished by MS analysis.

An experiment was performed to determine the optimal method of enriching the atmosphere surrounding rice seedlings. Replacing a gas sample, taken from a tube containing the seedling, with an equivalent volume of $^{15}N_2$ resulted in maintaining the TRIA induced, total N response. When the $^{15}N_2$ sample was injected with no previous gas withdrawal the response was inhibited (Table 10).

Table 10. A comparison of methods for $^{15}N_2$ atmosphere enrichment of TRIA treated rice seedlings.

^{15}N Enrichment method	Treatment TRIA (100 µg/L)	Total N (mg/plant)	Total N (mg/g dry wt)
Replace vol of atm with eq vol $^{15}N_2$	-	3.08	39.4
	+	3.60**	43.0**
Addition of $^{15}N_2$ to atm	-	3.01	39.3
	+	3.08	40.7

**F value for comparison with control significant at the .01 level.

The slight positive pressure imposed upon the seedling with the latter method may have caused the inhibition. The replacement procedure was utilized in all atmosphere enrichment experiments of this type.

The first enrichment experiment consisted of three treatments and six blocks in a randomized complete block design. Nineteen day-old rice seedlings were handled and gassed as previously described. A highly significant increase in dry weight was evident after 40 min incubation (Table 11).

Table 11. Dry weight change of TRIA treated rice seedlings in an 8.5% $^{15}N_2$ enriched atmosphere.

Time (min)	TRIA (100 µg/L)	Dry wt (mg/plant)
0	0	59.4
40	-	59.3
40	+	62.2**

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

To obtain sufficient sample for MS analysis the replicates were combined into three blocks. Although the apparent increase in total N due to TRIA was 14%, the increase in ^{15}N was only 4% and neither difference was significantly higher than the controls (Table 12).

Table 12. Total N and ^{15}N content of TRIA treated rice seedlings exposed to an 8.5% $^{15}N_2$ enriched atmosphere.

Time (min)	TRIA (100 μg/L)	Total N (mg/plant)	Total N (mg/g dry wt)	^{15}N (atom %)
0	0	2.10	35.3	0.375
40	-	2.28	38.2	0.374
40	+	2.59	41.3	0.390

The previous experiment was repeated and the samples were sent to another lab for ^{15}N analysis. After 40 min incubation, the TRIA treated plants gained in dry weight and total N over controls (Table 13). The increase was characterized by a gain in plant N (mg/plant) rather than concentration N (mg/g dry wt).

Table 13. Growth response, total N and ^{15}N content of TRIA treated rice seedlings exposed to an 8.5% $^{15}N_2$ enriched atmosphere. (Analysis conducted by R. P. Hauck, Division of Agricultural Department, Tenn. Valley Authority, Muscle Shoals, AL 35660).

Time (min)	TRIA (100 μg/L)	Dry wt (mg)	Total N (mg/plant)	Total N (mg/g dry wt)	^{15}N (atom %)
0	0	60.3	2.23	36.9	0.362
40	-	59.3	2.21	37.3	0.362
40	+	65.6**	2.39*	36.5	0.361

*,**F value for comparison with controls significant at the .05 and .01 levels, respectively.

If the apparent 7.5% increase had come exclusively from N_2 in the enriched atmosphere then the atom % ^{15}N in the TRIA treated plants should have increased to 0.978% from natural abundance. No increase in atom % ^{15}N was evident in any of the treatments (Table 13). It was apparent that atmospheric N_2 did not provide the N in the TRIA induced N response of intact seedlings.

Earlier results with Argon replacing N_2 revealed the need for the presence of N_2 to maintain the in vitro total N response. Therefore, an enrichment experiment was constructed utilizing corn extracts (Table 14).

Table 14. Total N and ^{15}N content of the supernatant from TRIA (1000 µg/L) treated corn leaves exposed to a 13% $^{15}N_2$ enrichment atmosphere.

Time (min)	Total N (mg/system)	(mg/g dry wt)	^{15}N (atom %)
0	28.19	86.5	0.365
120	29.51**	90.5**	0.366

**F value for comparison with control significant at the .01 level.

The concentration of N in the TRIA extracts increased significantly over the 2 h incubation period. Had this increase come directly from N_2 in the enriched atmosphere, the 2 h sample should have increased to 0.930 atom % ^{15}N. However, this sample remained at natural abundance.

The atmospheric substitution and enrichment experiments did not account for N dissolved in the solutions. Due to the high solubility of N_2 in water (17.8 mg/L), the possibility remained that there was sufficient N_2 dissolved in these solutions to contribute directly or indirectly in the TRIA response.

To test this possibility, a 40 min atmospheric substitution experiment was constructed. The shoots from treated 18-day-old rice seedlings were placed in test tubes containing half-strength Hoagland's solution. The tubes were stoppered and flushed with the appropriate gas mixtures. As in previous experiments, the concentration of CO_2 and O_2 were kept at normal levels while argon replaced N at an equivalent concentration. The concentration of N within the TRIA treated seedlings increased significantly regardless of the presence of atmospheric N_2 (Table 15). Since the experiment involved shoots only, the role of dissolved N_2 in the response was probably negligible.

Depletion and Distribution Studies

Depletion experiments were undertaken to test the hypothesis that TRIA was hastening the metabolism of contaminant sources of N. These compounds could affect both the atmosphere or nutrient solution. For example, N from nitrous oxide (N_2O) is readily metabolized by, and incorporated into, reduced N fractions of plants (38). The reactivity of N_2O in aqueous solutions results in the formation of nitrate and nitrite ions. Although normal atmospheres contain very little N_2O (0.2 μg/L to 0.5 mg/L) it is possible that the TRIA treated plants are utilizing it in combination with other unknown N-compounds possibly arising as a result of microbial activity within the nutrient solution. Depletion experiments permit the detection of any N utilization during an experiment on the basis of dilution of ^{15}N prelabelled plant material.

Both the *in vitro* and whole plant systems were utilized in these tests. The first experiment entailed culturing 17-day-old rice seedlings on half-strength Hoagland's solution containing 6 mM $K^{15}NO_3$ (5.3

Table 15. Growth response and total N content of TRIA treated rice shoots in a normal and argon substituted atmosphere.

Time (min)	TRIA (100 μg/L)	Atmosphere	Dry wt (mg/shoot)	Total N (mg/shoot)	(mg/g dry wt)
0	0	--	28.3	1.30	45.9
40	-	Normal	29.0	1.33	46.0
40	+	Normal	28.1	1.36	48.5**
40	+	Ar replacing N_2	29.9	1.45	48.2**

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

atom %) for 4 days prior to TRIA treatment. At the 21-day-old stage the plants were treated and placed in a growth chamber for 80 min after which the shoots were analyzed for total N and atom % ^{15}N. An increase in dry weight and total N was evident in the TRIA treatment at the end of the incubation period (Table 16). The gain in N occurred at a faster rate than the gain in dry weight as was apparent from the significant concentration increase (mg/g dry wt). Had the 16% increase in shoot N come from a nonlabelled, contaminating source of N, the atom % ^{15}N in the TRIA shoots should have fallen to approximately 2.20 atom %.

Table 16. Growth response, total N and ^{15}N content of TRIA treated rice seedlings grown on $K^{15}NO_3$ (5.3 atom %) for 4 days prior to treatment.

Time (min)	TRIA (100 µg/L)	Dry wt (mg/shoot)	Total N (mg/shoot)	Total N (mg/g dry wt)	^{15}N (atom %)
0	0	56.0	2.37	42.6	2.43
80	-	56.1	2.55	45.2	2.52
80	+	60.3**	2.97*	49.3*	2.50

*,**F value for comparison with controls significant at the .05 and .01 levels, respectively.

No significant decline in the label was evident. From this, and earlier experiments, it was concluded that the N which appears to be incorporated into TRIA treated plants is not entering from outside the system. In fact, it must be arising from within the plant itself and may be an artifact of Kjeldahl analysis.

An <u>in vitro</u> corn depletion experiment was conducted utilizing $K^{15}NO_3$ to raise the atom % ^{15}N of the extracts above natural abundance. A preliminary test had shown that equivalent levels of KNO_3 added to the

incubation media had no significant effect on the total N or the TRIA response. A 5% increase in total N occurred after 120 min incubation of the extract (Table 17). This should have resulted in a significant reduction in atom % ^{15}N from the zero-time sample had N entered the system from a source having natural abundance. However, there was no significant change in atom % ^{15}N.

Table 17. Total N, ^{15}N and reducing sugar content of the supernatant from TRIA (1000 μg/L) treated, corn leaves incubated in media containing $K^{15}NO_3$ (5.3 atom %) for 2 h.

Time (min)	Total N (mg/system)	^{15}N (atom %)	Reducing sugars (mg/system)	Nitrate reduced (%)
0	29.80	0.805	23.95	96
120	31.32*	0.796	26.96*	99

*F value for comparison with control significant at the .05 level.

An increase in reducing sugars results in enhanced reduction of nitrate to ammonium during Kjeldahl digestion (32). If the apparent N increases were arising through this type of an artifact, the 120 min samples should have had higher ^{15}N values (Table 17). The differences in atom % ^{15}N between the two treatments was not significant.

The relatively high atom % ^{15}N values in Table 17 indicate that most of the $K^{15}NO_3$ was digested via the standard micro-Kjeldahl procedure. An assay of reducing sugars revealed a significant increase in the 120 min sample. A close correlation existed between the increase in $K^{15}NO_3$ recovered and the increase in reducing sugars. However, it must be stressed that % ^{15}N recovery was calculated utilizing the

total N values obtained from Kjeldahl. Any error due to possible artifacts in the Kjeldahl procedure would cause an apparent ^{15}N increase.

It was hypothesized that TRIA may stimulate the conversion of one or more pools of non-Kjeldahl detectable nitrogenous compounds to detectable forms within the rice seedlings. This could be accomplished through a direct stimulation or inhibition of N metabolism or indirectly through alterations of carbohydrate and reducing sugar pools within the plant.

A series of N distribution experiments utilizing intact, prelabelled ^{15}N rice seedlings were designed to reveal a TRIA induced movement of N between various soluble and insoluble pools on the basis of depletion. By differentially labelling soluble and insoluble fractions with ^{15}N, movement of N from one fraction to another could be detected and appropriate comparisons made between treated and control plants.

Diehl (10) maintains that N in many organic compounds, including various forms of protein, is present in higher valence states and, as such, is not detectable via Kjeldahl. A procedure was sought which would result in combining the soluble protein N with the insoluble N resulting in a single water insoluble fraction. It was found that drying rice seedlings at 65 to 70°C resulted in denaturation of the soluble protein rendering it relatively insoluble. Hence, the majority of plant protein could be separated from amino acids, nitrate and other soluble N compounds with relative ease. If TRIA was enhancing the conversion of non-Kjeldahl detectable N to detectable sources then the probability that the non-detectable form(s) existed in this water insoluble fraction would be great. Such a conversion might be detected

on the basis of depletion in the TRIA treated samples, assuming that non-Kjeldahl detectable nitrogenous compounds exhibit slower metabolic turnover rates.

Nineteen-day-old rice seedlings were cultured on 6 mM $K^{15}NO_3$ (5.3 atom %) enriched half-strength Hoagland's solution for 20 h prior to treatment. The plants were treated, placed in a growth chamber for 80 min, and harvested by separating roots from shoots and drying both at 65°C. The insoluble N was separated from the soluble N and analyzed for ^{15}N content.

TRIA increased the dry weight, total N, total insoluble N and μg ^{15}N. However, the atom % ^{15}N values in the TRIA seedlings were not decreased compared to the control (Table 18). The increase in μg ^{15}N was due to a larger pool of total N in the insoluble fraction. The apparent N increase may, therefore, be due to an artifact within our Kjeldahl detection system.

An experiment was conducted with 18-day-old rice seedlings utilizing similar prelabelling and fractionation techniques. The roots and shoots were ground separately through a Wiley mill. An aliquot of the shoots was added to the roots, for fractionation and N determination, based on the original root/shoot dry weight ratio. Depletion of ^{15}N could thus be monitored and related to the whole seedling.

A significant increase in dry weight, total water soluble N, μg ^{15}N per soluble fraction and total μg ^{15}N per plant was evident with the TRIA treatment (Table 19). The increase in μg ^{15}N was due to a TRIA induced increase in total soluble N as determined by Kjeldahl. The atom % ^{15}N values were variable and showed no significant trends.

Table 18. Growth response, N and water insoluble ^{15}N content of rice seedlings cultured on $K^{15}NO_3$ (5.3 atom %) for 20 h prior to treatment with TRIA.

Time (min)	TRIA (100 μg/L)	Dry wt (mg/shoot)	Total N (mg/shoot)	Total N (mg/g dry wt)	Total insoluble N (mg/shoot)	Total insoluble N (mg/g dry wt)	Insol ^{15}N (atom %)
0	0	56.7	2.27	40.1	1.90	33.6	0.808
80	-	54.6	2.19	40.1	1.83	33.4	0.859
80	+	57.7*	2.47**	42.9*	2.08*	36.0*	0.858

*,**F value for comparison with controls significant at the .05 and .01 levels, respectively.

Table 19. Total N and ^{15}N in the water soluble and insoluble fractions of rice seedlings grown on $K^{15}NO_3$ (5.3 atom %) for 20 h prior to treatment with TRIA.

Time (min)	TRIA (100 µg/L)	Dry wt (mg/plant)	Total Soluble N (mg/plant)	Total Insol N (mg/plant)	Atom % ^{15}N Soluble	Insoluble
0	0	58.1	0.68	1.37	1.441	0.874
80	-	58.9	0.66	1.39	1.483	0.963
80	+	62.3**	0.72*	1.50	1.468	0.948

*,**F value for comparison with controls significant at the .05 and .01 levels, respectively.

It was apparent that TRIA did not induce interconversions of nitrogenous compounds within or between the two fractions. Determination of nitrate remaining in the culture tubes at the conclusion of the 80 min incubation period indicated no differences in nitrate uptake between the two treatments. The increase in soluble N within the TRIA treated plants could not be accounted for and appeared to be an artifact due to TRIA and associated with the Kjeldahl analysis system. Clearly, the accuracy of these experiments was limited by attempting to remove variation in ^{15}N incorporation by blocking for size.

An experiment was conducting with the plants being lyophilized at the end of the 80 min test period. The shoot material was fractionated into water soluble and insoluble portions and the TCA precipitable protein was separated from the soluble fraction and detected by a modified Lowry procedure (2). The insoluble N, soluble protein N, nitrate N and total N were determined and ^{15}N values compared between soluble protein and insoluble fractions.

The TRIA plants significantly gained in shoot dry weight, total N, soluble protein N, free amino N and insoluble N after 80 min (Table 20). Data for the N content of the insoluble fractions was estimated by subtraction of the soluble N components from the total N. No significant change in nitrate N had occurred at the conclusion of the experiment. If more nitrate was being reduced during digestion because of an increase in carbohydrates, a significantly higher atom % ^{15}N would be expected. There was no significant change in atom % ^{15}N in any of the distribution experiments.

Table 20. Distribution of N and ^{15}N in the water soluble and insoluble fractions of rice seedlings enriched with $K^{15}NO_3$ in the nutrient solution for 20 h prior to treatment with TRIA.

Time (min)	TRIA (100 μg/L)	Dry wt (mg/shoot)	Total N (mg/shoot)	Total N (mg/g dry wt)	Free Amino N (mg/g dry wt)
0	0	36.7	1.51	41.1	1.33
80	-	34.6	1.41	40.9	1.36
80	+	38.6**	1.65**	42.8**	1.46**

Time (min)	TRIA (100 μg/L)	Soluble Protein N (mg/shoot)	Insoluble N (mg/shoot)	Atom % ^{15}N Soluble Protein	Atom % ^{15}N Insoluble
0	0	0.44	0.88	1.051	1.081
80	-	0.42	0.81	1.018	1.061
80	+	0.51*	0.94*	1.070	1.085

*,**F value for comparison with controls significant at the .05 and .01 levels, respectively.

CONCLUSIONS

Characteristic of the response to TRIA has been an increase in growth (dry wt) and apparent Kjeldahl detectable N. The apparent N increase has been established on both a concentration (mg/g) and total plant (mg/plant) basis. Atmospheric substitution and enrichment studies confirmed that the apparent *in vitro* N response is dependent upon N_2 which is not used as substrate for the increase. Conversely, the apparent N increase *in vivo* is independent of atmospheric N. *In vivo* and *in vitro* ^{15}N depletion studies established that the apparent increase in total N from TRIA treatment does not come from the environment. Studies with differentially enriched N fractions of rice could not detect a TRIA induced redistribution of ^{15}N within the seedling. The apparent increase in N appears to be an artifact due to TRIA and associated with the Kjeldahl analysis system. Direct influences of TRIA on Kjeldahl seem highly unlikely due to the low rates at which it is used and the fact that it is ubiquitous within the plant kingdom. More probable, is the possibility that it alters the metabolism of the plant favoring a slightly different chemical makeup which interferes with the Kjeldahl process.

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