Rapid Growth and Apparent Total Nitrogen Increases in Rice and Corn Plants following Applications of Triacontanol

N. Richard Knowles and Stanley K. Ries
Department of Horticulture, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Triacontanol (TRIA) increased fresh and dry weight and total reducible nitrogen (total N) of rice (Oryza sativa L.) seedlings within 40 minutes. Increases in total N in the supernatants from homogenates of corn (Zea mays L.) and rice leaves treated with TRIA for one minute before grinding occurred within 30 and 90 minutes, respectively. The source for the increase was investigated utilizing atmospheric substitution and enrichment and depletion studies with 14N. The increase in total N in seedlings was shown to be independent of method of N analysis and the presence of nitrate in the plants. Automated Kjeldahl determinations showing apparent increases in N composition due to TRIA were shown to be correlated with hand Kjeldahl, elemental analysis, and chemiluminescent analysis in three independent laboratories. TRIA did not alter the nitrate uptake or endogenous levels of nitrate in corn and rice seedlings. Enrichment experiments revealed that the total N increases in rice seedlings, in vivo, and in supernatants of corn homogenates, in vitro, are not due to atmospheric N2. TRIA increased the soluble N pools of the plants, specifically the free amino acid and soluble protein fractions. No differences in depletion or enrichment of 14N incorporated into soluble and insoluble N fractions of rice seedlings could be detected on an atom per cent 14N basis. The apparent short-term total N increases cannot be explained by current knowledge of major N assimilation pathways. TRIA may stimulate a change in the chemical composition of the seedlings, resulting in interference with standard methods of N analysis.

Evidence of the growth-promoting effect of long chain alcohols can be traced back to 1959, when Crosby and Vlitos (4) demonstrated enhanced elongation of Avena coleoptile sections. Several years later, TRIA (2) (a naturally occurring, 30-carbon, straight chain, primary alcohol [CH₃(CH₂)₃₀CH₂OH]) was shown to increase the dry weight, leaf area, and, apparently, the total N when applied to rice and corn at concentrations ranging from 1 to 1,000 μg/g (17). TRIA had no effect on nitrate reductase activity in corn or rice at concentrations required to induce apparent N increases (13).

TRIA-induced growth increases in rice proved to be independent of light conditions, and CO₂ concentration appeared to play a regulatory rather than a substrate role (2). Six-h dark responses were characterized by increases in dry weight, soluble and insoluble Kjeldahl N, and soluble carbohydrates.

Jones et al. (11) demonstrated that a chain length of 30 carbons with a terminal hydroxyl group was specific for TRIA's growth-promoting 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 (11). Thus, the primary alcohols, octacosanol (28-C) and tetracosanol (24-C), are excellent inhibitors of the TRIA response at equimolar rates. TRIA increased the protein content of tobacco cell cultures. The response involved an increase in cell number, indicating a stimulatory effect on the rate of protein synthesis (8).

Metabolic profiling of extracts from rice seedlings grown in nutrient media containing D₂O showed that TRIA increased the incorporation of deuterium into several α-amino acids and organic acids (5).

This study was initiated to reveal the source of the total N increase which occurs both in vivo and in vitro in TRIA-treated rice and corn seedlings and extracts.

MATERIALS AND METHODS

Preparation of Plant Materials. Rice seed (Oryza sativa L.), 'IR-8' or 'ESD 7-1' (University of California, Davis, CA), was surface-sterilized with a 0.1% (w/v) solution of mercuric chloride. The seeds were planted in 77-ml plastic cups containing Turface, a clay substrate (Wyandotte Chemical Co., Detroit, MI), and watered to saturation with distilled H₂O. Growth conditions were a 16-h photoperiod at 30°C and an 8-h night at 25°C with approximately 7.0 μw/cm² in the photosynthetically active region (400 to 700 nm), as measured at the top of the canopy with photometer (International Light, Inc., Newburyport, MA). At the 7- to 11-day-old stage, the seedlings were transplanted into 220-ml plastic cups containing 180 ml of quarter-strength Hoagland solution (pH 4.5) containing 3 mM nitrate N (9). Four seedlings were suspended in the solution by a foam rubber disc in the top of the cup, which was wrapped in aluminum foil to exclude light. Nutrient solutions were renewed every 2 to 3 days thereafter with half-strength Hoagland solution containing 6 mM nitrate N.

Field corn (Zea mays L.), 'Pioneer 3780,' was sown in 18-cm clay pots (eight seeds/pot) containing a sterilized mix of equal volumes sand and sandy loam soils. The pots were placed in the greenhouse with a night temperature maintained at 25°C and a day temperature averaging 30°C. After 7 days, the seedlings received a commercial soluble 20-20-20 fertilizer twice a week at a concentration of 1 g/l. All plants received fertilizer or fresh nutrient solution the night before an experiment took place.

Preparation of Treatment Solutions. Treatment solutions were prepared from pure TRIA (American Cyanamid, Princeton, NJ) by making up in stock solutions of 0.1 to 1.0 mg/g (w/w TRIA-Tween 20 (polyoxyethylene sorbitan monolaurate)). The amount of stock added to glass distilled H₂O was adjusted to achieve a final concentration of 0.1% (w/v) Tween 20 and 100 to 1,000 μg/l TRIA. Treatment solutions for the inhibition experiment were prepared by dissolving TRIA or tetracosanol plus TRIA in chloroform (1.0 mg/ml) and diluting aliquots with distilled H₂O containing 0.1% (w/v) Tween 20 to give a final concentration of 100 μg/l of each alcohol.

---

1 Michigan Agricultural Experiment Station Journal article No. 9637.
2 Abbreviation: TRIA, triacontanol.
In Vivo Assays. Rice seedlings, previously sorted and blocked for size, were selected for treatment utilizing a random number table. The treatments consisted of the following: (a) no treatment, seedlings were harvested at the beginning of the experiment; (b) control, seedlings were inverted and dipped in 100 ml of a 0.1% (w/v) Tween 20 solution; and (c) TRIA, seedlings were inverted and dipped in a similar solution containing 100 μg/l TRIA. In one set of experiments, the seedlings were inverted and dipped in a Tween 20 solution containing both TRIA and tetraconanol. The treatment solutions were renewed after treating each replicate, and the roots of each seedling were rinsed three times in distilled H2O before being placed in 25-× 200-mm incubation tubes containing 10 or 12 ml of half-strength Hoagland solution containing 1 mm N as (NH4)2SO4. Treated plants were placed back in a growth chamber at 30°C for 40 to 80 min.

Harvesting the seedlings was accomplished either by separating roots from shoots, weighing the shoots, and immediately solubilizing the shoots for automated Kjeldahl analysis (7), or by freezing the entire plant in a Dry Ice acetone bath followed by lyophilizing or drying in a forced air oven at 72°C. In experiments involving the analysis of total system N, the nutrient solutions, containing (NH4)2SO4, 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 system N via automated Kjeldahl analysis.

In Vitro Assays. in vitro experiments were used because this recently described cell-free system (10) offered an excellent tool for studying the fate of 15N in a closed system. For the tests, the youngest one to three leaves of 7- to 10-day-old corn were planted in an Erlenmeyer flask with a 1,000-μg/l TRIA solution for 1 min, drained, and ground with a pestle in a cold mortar (4°C) with 4 ml buffer for each g fresh weight of tissue (10). Rice was treated with a 100-μg/l TRIA solution and ground with 5 ml buffer for every g of leaf tissue. The resulting crude homogenates were squeezed through four layers of cheese cloth and centrifuged for 20 min (8,000 g for corn, 5,000 g for rice). The supernatant solution was added to cold incubation medium (1:2 v/v) and kept on ice until the initiation of an experiment. The incubation medium contained NADH, NADPH, ATP, MgCl2, oxalacetate, and α-ketoglutarate (10). An experiment was initiated by pipetting predetermined volumes of the supernatant into 25-× 200-mm test tubes or 50- to 125-ml Erlenmeyer flasks. Zero-time samples were taken for total N analysis (12 ml for 15N and 3 ml for Kjeldahl analysis), 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 lyophilized or digested directly for total N analysis.

Analysis of Total N. Total N was measured, utilizing the automated Kjeldahl procedure of Ferrari (7). The plant samples were solubilized by adding digestion mixture (4 ml digestion mixture for every 20 mg dry weight) and heating until the solutions cleared. The digestion mixture contained 1,800 ml H2SO4, 40 ml HClO4, 6 g SeO3, and 160 ml H2O. After cooling, the samples were diluted with distilled H2O (6 ml for every 4 ml of digestion mixture) and poured into plastic cups which were placed on an Auto-Analyzer (Technicon Instruments Corp., Tarrytown, NY) for automated Kjeldahl analysis. Ammonium was detected spectrophotometrically at 623 nm by an alkaline-phenanol color reaction. Ground wheat standards (4.34% moisture, 2.566% N) were analyzed with each experiment for calibration purposes. The total N content of the wheat was established utilizing standard orchard leaf reference material (code 1571, National Bureau of Standards, Washington, DC).

15N Atmosphere Enrichment. Rice seedlings were treated with test solutions and placed in 35-× 300-mm test tubes containing half-strength Hoagland solution (pH 4.5) with 1 mm N as (NH4)2SO4. The amount of 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 septa, through which 10 to 25 ml of the atmosphere surrounding the plant was replaced with an equivalent volume of 15N2 (98.98 atom %). After the incubation period, plants were lyophilized and ground in a Wiley mill (40-mesh screen; Arthur H. Thomas Co., Philadelphia, PA) with aliquots taken for total N and 15N analysis utilizing a VG Micromass M-622 isotope ratio mass spectrometer (Isogas Ltd., Astonway, Middlewich, Cheshire, UK).

For the in vitro tests, supernatants of corn leaf homogenates were pipetted (80 ml) into 250-ml Erlenmeyer flasks from which zero-time samples were removed. The flasks were stoppered, and 22 ml of the atmosphere within the flasks were replaced with 22 ml of 15N2. This resulted in a 13% 15N-enriched atmosphere in each flask. The flasks were incubated for 120 min at 25°C in a water-bath with gentle shaking. Similar size samples were taken at the conclusion of the incubation period for total N and 15N analysis. Total N samples were digested immediately for automated Kjeldahl analysis, and samples for mass spectrometric analysis were dried at 72°C.

15N Depletion and Distribution Analysis. Rice seedlings were sorted for size and suspended in 180 ml of half-strength Hoagland solution having 6 mM K 15NO3 (5.3 atom %) for 20 to 96 h prior to treatment with TRIA. Prelabeling for 96 h necessitated the addition of labeled Hoagland solution twice over the 4-day interval. Following this period, the seedlings in each cup were sorted again, randomized, and treated. Treated plants were placed in 25-ml Erlenmeyer tubes containing 6 to 10 ml of labeled or unlabeled Hoagland solution and incubated for 80 min in a growth chamber. Plants were harvested and the roots and shoots separated prior to lyophilization or oven drying at 72°C. Several tests had previously established that the drying method did not change the proportional difference between controls and treatments. The dried shoots were ground in a Wiley mill separately from the roots. Aliquots were taken for total N, soluble protein, free amino acid, nitrate, and 15N analysis.

Nitrogen distribution experiments involved separation of the plant material into various N fractions followed by 15N analysis of each fraction. An aliquot of the dried plant material was extracted in a mortar with a pestle with glass-distilled H2O (1 ml for every 10 mg of plant material). The resulting extract was centrifuged at 10,000g for 30 min, and the supernatant solution was set aside. The pellet was washed with an equivalent volume of water and centrifuged again. Both supernatants were reconstituted (10,000g for 30 min), and the pellets were combined by transferring to 125-ml Erlenmeyer flasks with 25 ml distilled H2O. The resulting supernatant solutions were also transferred to 125-ml flasks, and all fractions were dried at 72°C.

Lyophilized plant material was used for isolation of soluble protein from water soluble fractions. Separation into soluble and insoluble fractions was achieved as discussed previously, and the soluble protein was precipitated with 20% TCA. Isolation of the protein was accomplished by centrifugation (10,000g for 30 min), and the resulting protein pellet was transferred to 125-ml Erlenmeyer flasks and dried at 72°C. Micro-Kjeldahl procedures (3) were employed to recover 15N from each fraction for mass spectrometric analysis.

Quantitative determinations of soluble protein N, free amino N, and nitrate N were made either by assaying the supernatant solutions in the regular fractionation sequence or by utilizing aliquots of the dried plant material. Soluble protein N was determined by a modification of the Lowry et al. procedure (1). The methods of Rosen (19) and Lowe and Hamilton (15) were used to assay free amino N and nitrate, respectively.

Preparation of Samples for MS Analysis. The micro-Kjeldahl procedures of Black (3) were employed for the digestion and distillation of plant samples for MS analysis. Catalyst, consisting
of a 1:10:100 mixture of SeO₂, CuSO₄, and K₂SO₄, 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 to convert the organic N to (NH₄)₂SO₄.

The NH₃ was released by addition of 10 n NaOH to the digestion mixture prior to distillation. Eighty percent ethanol was run through the distillation apparatus between samples to prevent cross-contamination. The resulting free NH₃ was trapped in 10 ml of 0.1 n HCl and dried at 72°C, resulting in pure NH₄Cl crystals. The MS analysis of ¹⁵N was accomplished by adding 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.

**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 size category 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%. Orthogonal comparisons were utilized in separating treatment effects from controls. F tests 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 labeled Erlenmeyer flasks containing incubation media. Coefficients of variation in these tests ranged from 1 to 4%.

**RESULTS AND DISCUSSION**

**In Vivo and in Vitro Assays.** Rice seedlings gained in fresh weight and in total N within 40 min of exposure to TRIA (Table I, test 2). The increase in N paralleled the increase in fresh weight. Interpretation of the data was complicated, because the increase in total N was larger than the amount of N provided to the plants via the nutrient solutions (0.14 mg N).

To further define the role of the nutrient solution in the apparent N increases, the total N in both whole plants and nutrient cultures was analyzed. After 40 min of exposure to TRIA, total system and total plant N had increased 21 and 24% respectively (Table I, test 2). The increase in total system N was further evidence that TRIA enabled the plants apparently to accumulate N from an unknown source.

In a similar experiment, the activity of TRIA was completely inhibited by tetracosanol (Table II), as previously reported (11).

**Table I. Growth Response of Rice Shoots and Rice Seedlings to TRIA in 40 Minutes**

<table>
<thead>
<tr>
<th>Time</th>
<th>TRIA</th>
<th>Fresh weight</th>
<th>Total N</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µg/l</td>
<td>mg/shoot</td>
<td>mg/system mg/seedling</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>215</td>
<td>1.98</td>
<td>1.69</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>223</td>
<td>1.97</td>
<td>1.73</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>252*</td>
<td>2.20*</td>
<td>2.10*</td>
</tr>
</tbody>
</table>

* F value for comparison with controls significant at the 0.01 level.

**Table II. Growth Response and Total N Content of Rice Seedlings Exposed to TRIA or TRIA + Tetracosanol for 40 Minutes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry Weight</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg/l</td>
<td>mg/plant</td>
<td>mg/system mg/dry wt</td>
</tr>
<tr>
<td>Control</td>
<td>42.7</td>
<td>1.70</td>
</tr>
<tr>
<td>TRIA</td>
<td>41.2</td>
<td>1.83b</td>
</tr>
<tr>
<td>TRIA + tetracosanol</td>
<td>43.0</td>
<td>1.71</td>
</tr>
</tbody>
</table>

* Unit measure, mg plant N/g dry weight of plant (Hoagland N subtracted).

b F value for comparison with controls significant at the 0.01 level.

**Table III. A Comparison of Manual Versus Automatic Determination of N Content of Rice Seedlings Exposed to TRIA When Grown with NH₄⁺ as the Only Source of N**

<table>
<thead>
<tr>
<th>TRIA</th>
<th>Time</th>
<th>Dry weight</th>
<th>Manual</th>
<th>Automatic</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/l</td>
<td>min</td>
<td>mg/plant</td>
<td>mg/g</td>
<td>mg/g</td>
<td>mg/g</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>98</td>
<td>4.38</td>
<td>4.19</td>
<td>0.004</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>96</td>
<td>4.36</td>
<td>4.36</td>
<td>0.005</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>104</td>
<td>4.51</td>
<td>4.48</td>
<td>0.003</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
<td>116a</td>
<td>5.22a</td>
<td>5.36a</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* F value for comparison of 80-min treatment versus controls significant at 0.05 and 0.01 levels, respectively. The correlation coefficient (r) between manual and automatic analysis was 0.988, significant at 0.05 level.

The total N content of the plants and plant-nutrient culture systems following 40-min treatment with TRIA was 12% and 7% higher, respectively, than that in the control or the TRIA + tetracosanol treatment. The total N increases apparently are unique to TRIA applied alone.

A similar increase in total N content of the supernatant of extracts from TRIA-treated corn leaves was evident over a 2-h incubation period. The concentration of total N in the 8,000g supernatant significantly increased from 74.7 mg/g in the zero-time control to 81.5 mg/g after 30 min. The N concentration was 81.8 and 82.9 mg/g after 60 and 120 min, respectively. Previous research (10) has shown that treating the leaves with control solution has no effect on the total N content of the supernatant from either corn or rice. Apparently, TRIA also enabled a source of N to accumulate in the in vitro system.

Several studies were conducted to establish whether or not the analytical method for N or the nitrate content of plants was responsible for the apparent N increase in TRIA-treated plants.

Plants were grown with only ammonium as a source of N prior to treatment, and then the same plants were analyzed by both the automatic procedure and the manual procedure for Kjeldahl determination. The results of the two methods were closely correlated, although there was more variation in the manual procedure (Table III). The increase from TRIA was clearly not due to a reduction of nitrate in the Kjeldahl procedure, because these ammonium-grown plants contained little or no nitrate (Table III).

Another rice test was analyzed for several elements including N by the micro-Kjeldahl procedure of Galbraith Laboratories (Knoxville, TN). These plants were also analyzed at Michigan

---

*Unit measure, mg plant N/g dry weight of plant (Hoagland N subtracted).

b F value for comparison with controls significant at the 0.01 level.*
State University (East Lansing, MI). The Galbraith results were highly correlated (r = 0.88**) with the Michigan State University results. Both showed increased total N from TRIA-treated plants based on N concentration or N per plant. The N results shown in Table V were obtained by manual Kjeldahl procedures in the Tennessee Valley Authority laboratory, Muscle Shoals, AL.

To further check the automatic micro-Kjeldahl procedure, it was compared with N analyses of the same samples by F. M. Ditri (Institute of Water Research, Michigan State University) using a Perkin-Elmer 240 elemental analyzer. Again, the results were similar (Table IV). This instrument combusts the sample to oxides of N which are catalytically converted to N₂ for volumetric detection. The plants from another test were analyzed with an Antek Model 707 nitrogen analyzer by Antek Instruments Inc. (Houston, TX). The results were correlated (r = 0.77**) with the Michigan State University automatic Kjeldahl procedure. This procedure determines total chemically bound nitrogen by pyrolysis, and detection is done by the chemiluminescent principle.

**N Atmosphere Enrichments. We hypothesized that the source for the rapid accumulation of N for TRIA treatments in both the in vivo and in vitro systems was the atmosphere. This becomes feasible when considering the possibility that TRIA may stimulate N₂ fixation by phytoplankton bacteria (12). The resulting reduced N would then be available for direct ammination within the plants. Bacteria in the leaves of Douglas fir (Pseudotsuga menziesii Franco) fixed a maximum of 5.2 mg N per g leaf N over a 1-month period. Similarly, Lovett and Sagar (14) demonstrated the presence of free-living N₂-fixing bacteria in the leaves of Commelina sativa (L.) Crantz. The apparent increases in total N content of plants due to TRIA are at least one magnitude higher than are these values. However, these estimates were based on research performed in natural environments; no attempt was made to maximize rates of fixation by optimization of growth conditions or by chemical stimulation.

To test the hypothesis that atmospheric N₂ was the source of additional N in TRIA-treated plants, the atmosphere surrounding the seedlings and in vitro supernatants was enriched with ¹⁵N₂. After a 40-min incubation period, TRIA had increased both the dry weight and the total N content of rice seedlings (Table V). The total N increase followed the gain in plant dry weight (mg/plant). If the apparent 7.5% increase had come exclusively from N₂ in the enriched atmosphere, the atom per cent ¹⁵N in the TRIA-treated plants should have increased from natural abundance (0.362%) to approximately 0.978%. No change in atom per cent ¹⁵N was apparent; thus, atmospheric N₂ was not the source of the increased N in TRIA-treated plants.

An atmosphere enrichment experiment utilizing the supernatants from TRIA-treated corn leaves showed a 5% increase in the concentration of N after a 2-h incubation period (Table VI). For

Table IV. Comparison of Kjeldahl Analysis with Analysis by Perkin-Elmer Elemental Analyzer on Rice Seedlings Treated with TRIA

<table>
<thead>
<tr>
<th>Time</th>
<th>TRIA Treatment</th>
<th>Dry Weight</th>
<th>Method of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µg/l</td>
<td>mg/shoot</td>
<td>Kjeldahl</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>56.7</td>
<td>2.27</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>54.6</td>
<td>2.19</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
<td>57.7*</td>
<td>2.47*</td>
</tr>
</tbody>
</table>

* F value for difference from controls significant at 0.05 and 0.01 levels, respectively. The correlation coefficient (r) is 0.999 (significant at 0.01 level) between the two methods.

Table V. Growth Response, Total N, and ¹⁵N Content of TRIA-Treated Rice Seedlings Exposed to a 8.5% ¹⁵N₂-Enriched Atmosphere

Analyses were carried out with lyophilized plants. Data are means for six replicates.

<table>
<thead>
<tr>
<th>Time</th>
<th>TRIA Treatment</th>
<th>Dry Weight</th>
<th>Total N*</th>
<th>¹⁵N</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µg/l</td>
<td>mg</td>
<td>mg/plant</td>
<td>mg/g dry wt</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>60.3</td>
<td>2.23</td>
<td>36.9</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>59.3</td>
<td>2.21</td>
<td>37.3</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>65.6*</td>
<td>2.39*</td>
<td>36.5</td>
</tr>
</tbody>
</table>

* Analyzed by R. P. Hauck, Division of Agricultural Development, Tennessee Valley Authority, Muscle Shoals, AL.

* F value for comparison with controls significant at the 0.05 and 0.01 levels, respectively.
this increase to come directly from N$_2$ in the enriched atmosphere, the 2-h sample should have increased to 0.930 atom per cent $^{15}$N. Inasmuch as $^{15}$N remained at the level of natural abundance, the N$_2$ present in the atmosphere above the supernatants was not incorporated.

15N Depletion and Distribution. Depletion experiments were conducted to test the hypothesis that TRIA accelerated the metabolism of contaminant sources of N in the atmosphere, in nutrient solutions, or in supernatants. For example, N from nitrous oxide (N$_2$O) is readily metabolized by, and incorporated into, reduced N fractions of plants (18). N$_2$O in water may form nitrate and nitrite ions. Although normal atmospheres contain very little N$_2$O (0.2 to 0.5 µg/l), TRIA-treated plants could utilize it together with other unknown N compounds arising from microbial activity within the nutrient solutions. Depletion experiments permit the detection of any source of N entering the plant during an experiment on the basis of dilution of $^{15}$N prelabeled plant material.

Rice shoots were labeled with $^{15}$N for 96 h prior to treatment and then treated with TRIA for 80 min. The gain in N (25%) occurred at a faster rate than did the gain in dry weight (7.7%), as was apparent from the 16% increase in N concentration (mg/g dry weight) (Table VII). Had the 25% increase in shoot N come from a nonlabeled, contaminating source of N, the atom per cent $^{15}$N in the TRIA-treated shoots should have been reduced appreciably. This did not occur, which indicates that the source of the apparent increase in N is not a contaminant from outside the system. Therefore, it must arise from within the plant itself. Similar depletion data were obtained with supernatants from corn leaves (13).

Nitrogen in many organic compounds, including various forms of protein, is present in higher valence states, and is only partly detectable via Kjeldahl analysis (6).

The hypothesis was proposed that TRIA stimulates the conversion of one or more pools of non-Kjeldahl-detectable nitrogenous compounds to detectable forms within the rice seedlings. This could occur directly via stimulation or inhibition of N metabolism or indirectly through alterations in carbohydrate and reducing-sugar pools within the plant. For example, reducing sugars have been found to increase following TRIA application (10), and there is evidence that such compounds result in greater reduction of nitrate to ammonium during Kjeldahl digestion (16). Such a conversion might be detected on the basis of depletion of TRIA-treated samples, assuming that non-Kjeldahl-detectable nitrogenuous compounds exhibit slower metabolic turnover rates.

A series of N distribution experiments was designed to reveal a TRIA-induced movement of N between soluble and insoluble pools on the basis of depletion. By prelabeling soluble and insoluble plant fractions with $^{15}$N prior to treatment with TRIA in unlabeled nutrient solutions, movement of N from one fraction to another could be detected and appropriate comparisons made between control and treated plants.

In an 80-min distribution experiment, TRIA-treated plants gained in dry weight and in total soluble N compared with controls (Table VIII). The atom per cent $^{15}$N values for both the soluble and the insoluble fractions were variable and showed no significant trends. Thus, TRIA did not induce interconversions of nitrogenous compounds within or between the two fractions.

A similar distribution experiment showed that the TRIA-treated plants gained in dry weight, soluble protein N, free amino N, and insoluble N after incubation for 80 min (Table IX). No change in shoot nitrate N between control and treated seedlings could be detected at the conclusion of the experiment. If more nitrate was being reduced during digestion, because of a possible increase in carbohydrates, a significantly higher atom per cent $^{15}$N value would be expected. No change in atom per cent $^{15}$N values occurred in any of the distribution experiments.

**CONCLUSIONS**

TRIA characteristically increases growth (dry weight) and apparent detectable N. Both total N (mg/plant) and concentration of N (mg/g) may be increased. The concentration of N never decreased in plants treated with TRIA, even though the dry weight increased. 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. A TRIA-induced redistribution of $^{15}$N within the seedling could not be detected in studies with differentially enriched N fractions of rice. The apparent increase in N appears to be interference by TRIA to systems for N analysis. TRIA probably alters plant metabolism leading to compositional or chemical changes which interfere with the methods for detecting total N studied in this research.

**Acknowledgments**—We thank Mrs. Violet Wert for her skillful technical assistance and Dr. David Dilley for his suggestions in designing equipment for, and in conducting, the $^{15}$N experiments.

**LITERATURE CITED**

5. DEmITTL DL, CC SWEELEY, JJ JONES, SK RIES 1978 Analysis of H incorporation into plant metabolites in control and 1-triacontanol-treated rice seedlings. Proceedings of the Third International Conference on Stable Isotopes, Argonne National Laboratory, Chicago, IL
7. FERRARA A 1960 Nitrogen determination by a continuous digestion and analysis

---

**Table IX. Distribution of N and $^{15}$N in the Water-Soluble and Insoluble Fractions of Rice Seedlings Cultured on $^{15}$NO$_3$ for 20 Hours Prior to Treatment with TRIA**

Plants were treated and placed in 6 ml half-Hoagland solution containing 6 mM KNO$_3$. Seedlings were lyophilized prior to N analysis. Data are means of three observations.

<table>
<thead>
<tr>
<th>Plant Component</th>
<th>Time</th>
<th>TRIA µg/l</th>
<th>Dry weight mg/shoot</th>
<th>Total N mg/g dry wt</th>
<th>Free amino N mg/g</th>
<th>Soluble Insoluble N</th>
<th>Insoluble N atom %</th>
<th>$^{15}$N Soluble Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>36.7</td>
<td>1.51</td>
<td>41.1</td>
<td>1.33</td>
<td>0.44</td>
<td>0.88</td>
<td>1.051</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>34.6</td>
<td>1.41</td>
<td>40.9</td>
<td>1.36</td>
<td>0.42</td>
<td>0.81</td>
<td>1.018</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
<td>38.6$^b$</td>
<td>1.65$^b$</td>
<td>42.8$^b$</td>
<td>1.46$^b$</td>
<td>0.51$^b$</td>
<td>0.94$^b$</td>
<td>1.070</td>
</tr>
</tbody>
</table>

$^a,b$ F value for comparison with controls significant at the 0.05 and 0.01 levels, respectively.