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PHYTOTOXICITY OF HERBICIDES

Reduction of 3-Amino-1,2,4-triazole Phytotoxicity in Tomato Plants

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The phytotoxicity of 3-amino-1,2,4-triazole was studied using tomato plants to determine the efficacy of selected compounds in reducing growth and chlorophyll toxicities. When the concentration of 3-AT was 1×10^{-4}M, equimolar solutions of certain purine precursors simultaneously applied caused a reduction of 3-AT growth inhibition; but this did not occur when all concentrations were five times this amount. Adenine, hypoxanthine, and guanine and their ribosides acted like the purine precursors. Riboflavin, FMN, and FAD reduced not only 3-AT growth inhibition at 5×10^{-4}M, but also the inhibition of chlorophyll formation. A hypothesis of the mechanism of 3-AT phytotoxicity is presented.

The compound 3-amino-1,2,4-triazole (3-AT), also known as aminotriazole and Amitrol, is an effective defoliant and herbicide. Green plants, treated with sublethal dosages of 3-AT, manifest toxic effects in two ways—growth of embryonic tissues is inhibited, and leaves developed after treatment with 3-AT are devoid of chlorophyll. In addition, albinistic leaves in several species lack chloroplasts (*16, 18*). Thus, it may be concluded that 3-AT mainly affects developing rather than mature tissues.

Evidence related to the biochemical mechanism is limited. Possibly, the interaction of 3-AT with several metals to form stable complexes is responsible for the toxicity symptoms; however, Hall, Johnson, and Leinweber (*6*) found that plants inhibited by 3-AT were not benefited by treatment with excess metal ions.

The structural similarity between 3-AT and pyrrole makes it attractive to speculate that the phytotoxicity of 3-AT is in some way related to porphyrin or porphyrinlike substances. In support of this hypothesis, Heim, Appleman, and Pyfrom (*7*) and Pyfrom, Appleman, and Heim (*16*) have shown that crystalline catalase activity and catalase activity of plants treated with 3-AT are depressed. Likewise, Margoliash and Novogrodsky (*12*) have shown that 3-AT causes a complete inhibition of crystalline preparations of liver and erythrocyte catalase in the presence of hydrogen peroxide. On the other hand, Bogorad (*1*), in his studies on the enzymatic synthesis of porphyrins from porphobilinogen (PBG), found that 3-AT up to $5 \times 10^{-3}M$ had no adverse effects on a system containing PBG and porphobilinogen deaminase.

The present report provides further evidence concerning the biochemical effect of 3-AT inasmuch as the 3-AT phytotoxicity was reduced by appropriate physiological compounds.

Procedure

Chemicals. Technical grade 3-amino-1,2-4-triazole was supplied by the American Cyanamid Co. and was recrystallized twice before use. 4-Aminoimidazole (4-AI) was prepared in aqueous solution according to the method of Rabinowitz (*17*).

Biological Assay. The effect of 3-AT on plants was studied by the observation of growth and chlorophyll development of the apparent first leaf of tomato plants subsequent to treatment of the growing tip of the plant with 3-AT and other compounds. Tomato plants, *Lycopersicum esculentum* Mill. var. Bonny Best, were grown in the greenhouse at 60° to 85° F. in vermiculite watered periodically with a complete nutrient solution (*8*) until the plants had reached the 8- to 12-leaf stage.

The growing tips of plants at this stage were treated with various chemical solutions. To accomplish this, the apparent third leaf was excised and a powder funnel was fitted around the terminal 3 cm. of the plant by means of a split rubber stopper sealed to the stem with anhydrous lanolin. The terminal leaflets of the apparent first and second leaf were removed to afford a higher proportion of juvenile tissue for treatment and to provide well defined points from which growth measurements could be made (Figure 1). With such an arrangement, the growing tip and the first two leaves could be totally submerged in the desired solution for a given period, at the termination of which the solution could be siphoned away. After treatment, the plants were maintained under normal greenhouse conditions.

The period of time during which the plant tip was exposed to the 3-AT solution is designated in this paper as treatment time. The inhibition of growth caused by treatment with $10^{-4}M$ 3-AT rapidly approached a value of about 50% for a treatment time of 12 hours. When the effect of two compounds on the same plant was tested, they were applied simultaneously in the same solution, as experience indicated that the inhibition caused by treatment with 3-AT could be partially reduced merely by an im-

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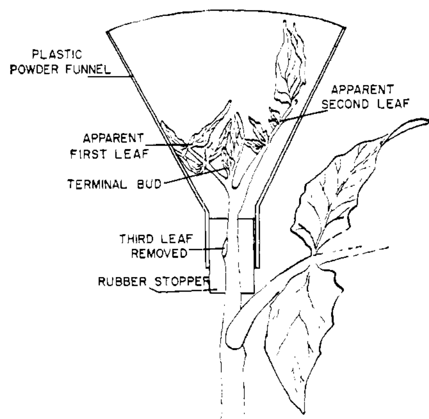

Figure 1. Diagram of setup used in the biological assay

Table I. Efficacy of Selected Compounds in Reducing 3-AT Growth Inhibition

			Growth Increment as % of Control			
				3-AT Plus Compound Indicated		
Expt. No.	No. of Plants	Growth Increment of Controls × 100	3-AT $10^{-4}M$	Adenine $10^{-4}M$	Guanine $10^{-4}M$	Hypoxanthine $10^{-4}M$
19[a]	8	305	82.5		94.8[b]	
10	5	471	80.0			87.6
18[a]	7	302	73.2		91.1[c]	98.5[c]
20	8	479	70.3	101[c]		93.6[c]
11	6	424	70.2		85.5[b]	
21	7	567	67.8	86.4[c]		
27[d]	10	415	66.6	55.5		54.6
12	5	730	66.5			91.2[c]
5	4	371	59.9			62.4
23[d]	8	635	59.8	53.7	60.9	61.6
22	8	427	50.3		44.2	
				Adenosine $10^{-4}M$	Guanosine $10^{-4}M$	Inosine $10^{-4}M$
24	8	535	83.1	95.1[b]	94.0[b]	104[c]
10	6	471	80.0			92.5[b]
4[e]	4	256	78.4			93.3[c]
25[f]	8	537	78.0	97.6[c]	90.9[b]	99.2[c]
3	6	363	70.0			79.9[c]
12	5	730	66.5			94.1[c]
				4-AI[g] $10^{-4}M$	4-A-5-IC[g] $10^{-4}M$	
24	8	535	83.1	101[c]		
19[a]	8	305	82.5	96.1[b]		
25[f]	8	537	78.0	95.3[c]		
26[h]	10	525	75.8	86.6[b]		
11	6	424	70.2		80.7	
27[d]	10	415	66.6	54.4	54.4	
16	10	349	64.6	96.9[c]		
26[i]	10	525	54.6	70.6[c]		
15[j]	12	512	45.6		54.2[c]	

[a] 6-hr. treatment.
[b] Mean of growth increment significantly different at 5% level from that of 3-AT alone.
[c] Mean of growth increment significantly different at 1% level from that of 3-AT alone.
[d] All concns. $5 \times 10^{-4}M$.
[e] Experiment of 4 days.
[f] All concns. $2.5 \times 10^{-4}M$.
[g] Purine precursors.
[h] 6-hr. treatment; all concns. $2 \times 10^{-4}M$. [i] 6-hr. treatment; all concns. $4 \times 10^{-4}M$.
[j] 12-hr. treatment.

mediate second treatment with an aqueous solution.

For any one set of plants the growth response was proportional to the concentration of 3-AT. For example, in one experiment with $10^{-5}M$, $10^{-4}M$, and $10^{-3}M$ 3-AT the growth was 90, 70, and 35% of the controls, respectively. However, as is evident from the data reported, the per cent inhibition of growth caused by $10^{-4}M$ 3-AT in different experiments was variable. The plants were less inhibited at high greenhouse temperatures—i.e., greater than 90° F.—and light quantity might have contributed to the noted variation.

The standard assay involved treatment of tomato plants with water for controls, a $10^{-4}M$ 3-AT solution, and a $10^{-4}M$ 3-AT solution containing equimolar amounts of one or more compounds to be assayed for efficacy in reduction of toxicity. None of the chemicals used showed any visible reaction with 3-AT at the concentrations employed. Treatment time was usually 3 hours; variations from this procedure are noted where applicable.

The effect on growth of 3-AT and of 3-AT plus the compound to be tested was quantitatively evaluated as follows: The length of the apparent first leaf was measured at the start of the experiment. For most of the experiments, this value was 10 to 15 mm. Six days after treatment, a second measurement was made. From these data, growth increments were calculated as follows:

$$\text{Growth increment} = \frac{\text{length of first leaf at 6 days} - \text{length of first leaf initially}}{\text{length of first leaf initially}}$$

These values were then related to that of the control plants as follows:

$$\% \text{ of control} = \frac{\text{growth increment of treated plant}}{\text{growth increment of control plant}} \times 100$$

To evaluate the statistical significance of the difference between the growth of the plants treated with 3-AT only and that of plants treated with 3-AT plus a second compound, the *t* test was applied to the means of the respective growth increments. The significant differences found are indicated in the tables. The means of the growth increments of plants treated solely with guanine, hypoxanthine, inosine, 4-aminoimidazole (4-AI), 4-amino-5-imidazolecarboxamide (4-A-5-IC), glutamine, histidine, and aspartic acid did not differ significantly at the 5% level from those of the control plants.

The first leaf and terminal bud 6 days after treatment were assayed for chlorophyll content relative to the water-treated control plants. After extraction of the pigment according to Petering, Wolman, and Hibbard (*15*), the procedure of Milner *et al.* (*14*) was used. From these data, expressed as Klett units per gram of fresh weight, the relative chlorophyll content was calculated as per cent of the control plants.

Results and Discussion

Reduction of 3-AT Growth Inhibition by Purines. Table I indicates that the growth inhibition of the tomato leaf caused by 3-AT was significantly reduced when adenine, guanine, or hypoxanthine was supplied simultaneously with the inhibitor, although reduction did not occur in all experiments. Nevertheless, except for experiment 10, the data are consistent with the interpretation that the purines tested resulted in significant partial reduction of growth inhibition caused by 3-AT, provided the inhibition effected by 3-AT alone was not too severe. When the concentrations were all $5 \times 10^{-4}M$, as in experiment 27, the purines were ineffective in reversing 3-AT growth inhibition.

In accordance with these observations the effect of various ribosides was of interest. Table I further indicates that under the assay conditions employed adenosine, guanosine, and inosine also significantly reduced growth inhibition caused by 3-AT.

As purines and their ribosides were effective for minimizing growth inhibition, a number of precursors known to be involved in purine biosynthesis in other systems were assayed. The data of Table I indicate that 4-aminoimidazole and possibly 4-amino-5-imidazolecar-

boxamide were effective agents. On the other hand, comparable experiments with glycine, histidine, aspartic acid, and glutamine failed to demonstrate that these amino acids reduce growth inhibition caused by 3-AT.

The partial reduction of growth inhibition by purines and metabolically related compounds indicates that 3-AT may in some manner interfere with normal purine metabolism. Disruption of purine metabolism could logically account for decreased growth, as the relationship of nucleic acid and protein synthesis is well established.

Reduction of 3-AT Growth Inhibition by Riboflavin. For some organisms, purines not only stimulate riboflavin production (*11*) but also become incorporated into the riboflavin molecule (*9, 13, 19*). This metabolic relationship suggests that an effect comparable to that of purines on 3-AT inhibition might also be produced by riboflavin. The data of Table II indicate that riboflavin as well as FMN and FAD is effective in reducing growth inhibition caused by 3-AT. In these experiments, even in series showing strong inhibition by 3-AT, there is conclusive evidence for significant reduction of toxicity.

Reduction of 3-AT Chlorophyll Inhibition by Riboflavin. Although the purines and related compounds indicated in Table I were effective in partially reducing the inhibitory effect of 3-AT on growth, none of these chemicals caused any visible reduction of chlorophyll inhibition. However, Table II indicates, for tomato tips treated simultaneously with flavin mononucleotide (FMN) and 3-AT, a very marked reduction of the chlorophyll inhibition. FMN alone did not influence the chlorophyll content of plants not treated with 3-AT. Riboflavin and flavin adenine dinucleotide (FAD) also reduced in a similar manner the degree of chlorophyll inhibition caused by 3-AT. Even plants under conditions which had as low as 25% or less of the chlorophyll found in control plants when treated with 3-AT alone formed as much as 75% or more of the normal chlorophyll content when treated with 3-AT solutions including riboflavin derivatives.

Rogers' work indicates that the toxicity of 3-AT is not related to porphyrin or chlorophyll synthesis itself (*18*). Also, Hall, Johnson, and Leinweber (*6*) have reported that chlorophyll synthesis occurred in etiolated cotton cotyledons which had been sprayed with 3-AT. Furthermore, studies in the authors' laboratory have failed to reveal any inhibition by 3-AT of porphyrin and bacteriochlorophyll production by *Rhodopseudomonas spheroides* cultured under the conditions of Lascelles (*10*). The obvious inhibition of growth and of normal plastid formation in newly formed 3-AT treated tissues points rather

Table II. Efficacy of Riboflavin and Its Derivatives in Reducing 3-AT Growth and Chlorophyll Inhibition

			Growth Increment as % of Control				
			3-AT Plus Compound Indicated				
Expt. No.	No. of Plants	Growth Increment Controls × 100	3-AT $5 \times 10^{-4}M$	Riboflavin $5 \times 10^{-4}M$	FMN $5 \times 10^{-4}M$	FAD $5 \times 10^{-4}M$	Riboflavin + ATP $5 \times 10^{-4}M$
36	8	467	70.4		80.2[a,b]		
32	9	529	66.9	87.9[c]			98.7[c]
35	10	649	69.4	93.1[c]	87.6[c]	87.2[c]	
37	10	381	68.6		91.9[a,c]		
34	5	492	60.1	83.3[c]			
39	8	389	53.6	68.1[c]	86.5[c]		77.3[c]
40	10	444	52.6	90.8[c]			101[c]
			Relative Chlorophyll Content as % of Control				
35			82.0	97.5	96.0	95.1	
34			54.0	85.7			
39			25.2	59.0	93.7		84.7
36			22.9		82.2[a]		
32			20.8	74.8			78.0
37			17.5		85.1[a]		
40			11.6	76.5			91.2

[a] FMN was $5 \times 10^{-5}M$.
[b] See [b], Table I.
[c] See [c], Table I.

to the interpretation that the essential action of 3-AT is to disrupt the metabolism of purines and riboflavin and their derivatives.

The inhibition of plastid formation and consequently of chlorophyll synthesis by 3-AT would seem from the present study to result from the effectiveness of 3-AT in blocking the synthesis of certain metabolites, chiefly riboflavin, necessary for normal chloroplast development. A comparable effect on chloroplast development in albinistic *Euglena* has been reported by De Deken-Grenson (*2*), who noted that amino acids and yeast RNA selectively promote chloroplastic growth under conditions where growth was unaffected.

Additional data which will be reported shortly indicate that 3-AT inhibits riboflavin synthesis in the yeast *Eremothecium ashbyii*. Also, albinistic tissues of corn and pea plants treated with 3-AT show much less riboflavin than do tissues taken from comparable parts of untreated plants.

The inhibition of vitamin B_2 production is of interest with other studies which have shown that riboflavin content in tomato plants increases with high light intensity (*3*), long photoperiods (*4*), and high temperatures (*5*), factors which were not kept constant in these studies and which might have contributed to the variability of data observed.

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