

STUDIES ON THE EFFECT OF AMINOTRIAZOLE ON CHLOROPLAST DEVELOPMENT IN *PHASEOLUS RADIATUS* L.

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ABSTRACT

The effect of 3-amino-1, 2, 4-triazole (amitrole) on the development of chloroplast is studied by exposing dark grown seedlings of *Phaseolus radiatus* L. to light. The syntheses of chlorophyll and carotenoid pigments, as a function of chloroplast development, were found to be affected specifically. The inhibitory action of amitrole on carotenoid level is more pronounced than on chlorophyll content.

A few aminoacids, riboflavin and ethylene diamine tetra-acetate (EDTA) were found to effectively reverse the inhibitory effect of aminotriazole when administered concurrently. Likewise ferric and magnesium ions also nullified the toxic effect of this compound. A possible mode of action of this substance, in interfering with the development of chloroplast is discussed, in the light of the above observations.

INTRODUCTION

THE herbicide 3-amino-1, 2, 4-triazole is widely used as a defoliant and as a growth inhibitor. This compound is found to produce albinism—a state in which chloroplast development is completely inhibited—in higher plants without any marked effect on the cellular metabolism or the morphology of the leaves at sub-lethal concentrations. The extensive work carried out to understand its mode of action has been reviewed¹⁻². Though its primary site and mode of action in heterotrophic microorganisms is adequately explained, definite information with reference to its site and mechanism of action in higher plants—particularly a basis for its selective inhibition of the chloroplast development—seems yet to be understood. This paper reports our studies on the effect of this compound on the development of chloroplasts with special reference to the synthesis of chlorophyll and carotenoid pigments in the young leaves of *Phaseolus radiatus* L. seedlings under conditions of maximal chloroplast development.

MATERIALS AND METHODS

Plant Material

Phaseolus radiatus L. seedlings used in this study were raised on sandy loam in plastic trays for 7 days either in the dark or in light from a bank of fluorescent lamps, at an intensity of 4,200 lux. Only primary leaves of the seedlings were used throughout, for analytical studies. In greening experiments, the dark grown seedlings were pretreated, before exposure to light, with 5 and 10 mM aminotriazole for 24 hours by keeping their roots immersed in the test solutions.

Reversal of Amitrole Bleaching

In experiments designed to study the reversibility of amitrole bleaching, the 6-day old dark grown

seedlings were treated in the dark for 24 hours with solutions of equimolar (5 mM) concentrations of amitrole plus aminoacids, or other substances like metal ions, EDTA and riboflavin. After pretreatment in the dark for 24 hours, the seedlings were exposed to light for an additional 24 hours in the same solutions.

Experiments with Excised Leaves

To study the effect of amitrole on greening, two sets of leaves from 7-day old dark grown seedlings were pretreated in petri dishes with 5 mM amitrole and distilled water separately by floating in dark for 24 hours before transferring to light. Leaf samples were removed at indicated time intervals and their pigments estimated.

Estimation of Chlorophyll and Carotenoids

The pigments were extracted from 100 mg leaf samples by grinding in a tissue homogenizer with 80% aqueous acetone. The chlorophyll and carotene contents in the acetone extract were estimated spectrophotometrically according to the methods of Arnon³ and Goodwin⁴ respectively.

RESULTS

The effect of aminotriazole on the development of chloroplast in the leaves of dark grown *Phaseolus* seedlings on exposure to light is shown in Table I. Normally, the chloroplasts in *Phaseolus* seedlings take about 20 hours for the complete development as measured in terms of chlorophyll content. When fully developed, they contain about 2 mg of chlorophyll per gram fresh weight of the leaf. The chloroplast development was retarded by 25 and 50% respectively in 5 and 10 mM amitrole-treated seedlings. At concentrations higher than 10 mM, amitrole seemed to act as a non-specific toxin affecting root growth, leaf size, etc., and hence in

TABLE I

Effect of amitrole on the development of chlorophyll and carotenoid in dark grown seedlings of Phaseolus on exposure to light

Hours of illumination	Mgchl/gm fresh weight			μ g carotenoid/gm fresh weight		
	Control	AT	AT	Control	AT	AT
		5 mM	10 mM		5 mM	10 mM
0	0.10	0.10	0.30	6	5	5
2	0.27	0.22	..	14	12	9
4	0.57	0.41	0.25	26	22	13
6	0.84	0.58	0.45	40	29	20
12	1.71	1.17	0.97	80	43	29
16	1.90	1.50	1.11	100	48	33
20	2.00	1.53	1.18	120	52	35
24	2.03	1.53	1.20	126	60	35

TABLE II

Effect of amitrole on the chlorophyll synthesis in excised leaves

Two sets of excised leaves from 7-day old dark grown seedlings of *Phaseolus radiatus* were floated in distilled water and 5 mM amitrole separately and were illuminated with a bank of light. Leaf samples were removed at indicated time intervals and their chlorophyll content estimated.

Hours of illumination	μ g chlorophyll/gram fresh weight	
	Control	Amitrole
0	50	50
2	101	75
4	158	108
6	197	110
8	242	106
10	279	110
12	330	109
14	365	115

all our experiments, concentrations of less than 20 mM amitrole were employed.

The effect of amitrole on the chloroplast development in excised dark grown leaves exposed to light is shown in Table II. There was a rapid synthesis of chlorophyll in dark grown, excised leaves floated in distilled water during illumination. The chlorophyll content increased 7-fold within 14 hours of illumination. There was only a 2-fold increase during the same period when the leaves were

floated in 5 mM aminotriazole. This amounted to nearly 70% of inhibition as compared to control leaves. The effectiveness of amitrole in causing maximal inhibition of chloroplast development when applied directly to the leaves and not through the roots is obviously due to the limited absorption and translocation in the latter case.

The carotenoid synthesis in the leaves of amitrole-treated seedlings after exposure to light is compared with the leaves of control seedlings as shown in Table I. The carotenoid content reached the maximal level of 125 μ g per gram fresh weight in control leaves within 24 hours. The levels of carotenoid in amitrole-treated seedlings were, however, reduced to 60 and 35 μ g per gram fresh weight, depending on the concentration of amitrole administered. The effect of amitrole on carotenoid biosynthesis was more pronounced than that of chlorophyll synthesis.

Diverse metabolites like aminoacids, bases and vitamins were shown to annul or nullify the toxic effect of amitrole when applied concurrently with or later of its application in organisms like unicellular algae, yeast and bacteria which has led to the speculation that amitrole might interfere with their biosynthesis. In order to ascertain whether similar reversal of the inhibitory effect of amitrole on chloroplast development could be achieved, a systematic study was made with several compounds, that were shown to annul its effect by others, on their ability to reverse the bleaching effect of amitrole in the leaves of *Phaseolus* seedlings. The effect of equimolar concentrations of various metabolites in reversing the amitrole-induced inhibition of chlorophyll synthesis is summarised in Table III. Among the aminoacids tried, L-histidine, glycine and sodium succinate mixture (as substrates for δ -aminolevulinate synthesis), L-leucine and L-cysteine were found to reverse completely the inhibitory action of amitrole on chloroplast development; while the aminoacids like DL-glutamic acid, DL-serine and glycine were comparatively less effective in retarding the inhibition caused by amitrole. DL-phenylalanine, lysine and L-cystine, on the other hand, were found to have practically no effect. Riboflavin was the other substance tried and found to be effective in annulling the inhibitory action of amitrole.

Similarly, the effect of certain metal ions and the chelating agent, ethylene diamine tetra-acetic acid (EDTA) on reversing the effect of amitrole on chloroplast development as measured by chlorophyll content is shown in Table IV. Among the metal ions tried Fe^{3+} , Mg^{2+} were very effective in nullifying the action of amitrole while Ca^{2+} and Na^{+} had no effect at all. IDIA when applied along with amitrole annulled the inhibitory action of the compound completely.

TABLE III

Effect of various aminoacids on the inhibitory action of amitrole on the chlorophyll content of *Phaseolus* seedlings

Dark grown 6-day old seedlings were treated either with 5 mM amitrole separately or along with an equimolar concentration of aminoacids. The treated seedlings were kept in the dark for 24 hours. The pretreated seedlings were then exposed to light for a period of 24 hours and total chlorophyll and carotenoid levels estimated at the end of the period. The figures given are the average of three different experiments.

Treatment	Total chlorophyll $\mu\text{g/gm}$ fresh weight	% Control	Total carotenoid $\mu\text{g/gm}$ fresh wt.	% Control
None	1250	100.00	198	100.00
Amitrole	550	44.00	80	40.40
+Riboflavin	1263	102.54	200	101.00
+Glycine	1200	96.00	197	99.49
+Sodium succinate				
+L-Histidine				
+L-Leucine	1120	89.60	189	95.45
+L-Cysteine	1100	88.00	179	90.40
+DL-Serine	890	71.20	160	80.80
+DL-Glutamic acid	908	72.60	158	79.79
+Glycine	785	62.60	141	71.21
+DL-Alanine	540	42.80	86	43.33
+DL-Phenyl-alanine	533	43.20	83	41.91
+Lysine	510	40.80	80	40.40
+L-Cystine	500	40.00	76	38.38

DISCUSSION

Among the various herbicides which are known to inhibit the photosynthetic function of the leaves, amitrole was found to affect the chloroplast development specifically and completely without any significant effect on the rest of the cellular metabolism⁵. From the data presented here, it can be seen that when amitrole-treated dark grown leaves were exposed to light both chlorophyll and carotenoid developments are inhibited. Since the two pigments are not biosynthetically interrelated, the mechanism for the concurrent inhibition of both chlorophyll and carotenoids cannot possibly be due to the direct action of amitrole on their biosynthesis. This is substantiated by the fact that the enzymes involved in the biosynthesis of prophyrin or heme were not inhibited by amitrole under *in vitro* con-

TABLE IV

Effect of various metal ions on the inhibitory action of amitrole on the chlorophyll content of *Phaseolus* seedlings

The experimental details are as in Table III.

Treatment	Total chlorophyll $\mu\text{g/g}$ fresh weight	% Control	Total carotenoid $\mu\text{g/gram}$ fresh wt.	% Control
None	1250	100.00	198	100.00
Amitrole	550	44.00	80	40.40
+Ferric chloride (1 mM)	1272	102.54	195	98.98
+Magnesium chloride (1 mM)	1189	95.12	187	95.45
+Calcium chloride	545	43.60	83	41.91
+Sodium phosphate	505	50.50	81	40.90
+EDTA (0.5 mM)	1243	102.54	200	100.00

ditions⁶⁻⁸. Neither were there evidences for the accumulation of specific intermediates of chlorophyll in amitrole-treated cells. There was also no generalized reduction in the levels of all porphyrin containing compounds in amitrole-treated cells⁹.

Though accumulation of phytoene—a carotenoid precursor—in amitrole-treated cells is reported, it is not known whether it is the breakdown product of carotenoids¹⁰, or due to an inhibition of further conversion of this intermediate¹¹. Phytoene accumulation itself has not been confirmed by others. Among the various biochemical compositions compared between normal and amitrole-bleached cells, total lipid was found to be drastically reduced besides the pigments in amitrole-bleached cells¹². Considering all these facts, it is reasonable to conclude that the observed decrease in the levels of the two pigments may be due to interference in the development of the chloroplast structure preventing the accumulation of these pigments rather than interfering with their syntheses *per se*.

The reversal of amitrole inhibition of chloroplast development by a set of highly unrelated compounds such as a few aminoacids, riboflavin, purines and certain metal ions¹³ at equimolar concentration indicate that this herbicide is not affecting the chloroplast development either by interfering with the syntheses of these pigments or with the metabolism of these compounds directly. It is likely that these

compounds would complex with aminotriazole to a varying level and thereby either prevent its absorption into the cell or detoxify it or its derivatives inside the cell. In fact, it has been shown that amitrole forms complexes with proteins or amino-acids¹⁴⁻¹⁶.

Similarly, the fact that certain metal ions like Fe^{3+} and Mg^{2+} at equimolar concentrations could also reverse its adverse effects (Table IV), indicates that amitrole possibly acts as a chelator to interfere with the normal utilization of Fe^{3+} or Mg^{2+} in the synthesis of proteins like cytochromes or pigments which in turn would effect the formation of lamellar membrane. The evidence in favour of this comes from the fact that these ions were found to reverse the observed immediate inhibition of the photosynthetic and respiratory oxygen exchange reactions by amitrole¹². Apparently amitrole seems to be very specific in complexing with certain metal ions, since the calcium and sodium ions have no such reversal effect on the herbicidal action of amitrole (Table IV).

In conclusion it can be seen from the data presented here that the observed low levels of carotenoid and chlorophyll pigments are only an effect on the development of chloroplast structure rather than its direct interference with their biosynthesis. Similarly the reversal of amitrole inhibition by several organic compounds may be due to their ability to complex with amitrole thereby either preventing its entry into cells or detoxify it inside the cells. The fact that certain metal ions could also reverse the amitrole effect substantiates this possibility.

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