ABSTRACT

THE BASIS FOR ENHANCED PHYTOTOXICITY OF ATRAZINE-PHOSPHATE COMBINATIONS

by

Charles Frederick Stolp

This study confirmed the occurrence of an interaction between atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) and phosphate which produces a synergistic reduction in plant growth not due to increased herbicide uptake or altered mineral composition. The objective of this study was to find the basis of this interaction.

Of the five crop species examined, corn (Zea mays L.), peas (Pisum sativum L.), sorghum (Sorghum vulgare L.), and soybeans (Glycine max Merril) showed greater reduction in plant growth following atrazine treatment if high levels of phosphate were also present in the root treatment solution. Only in the case of peas was the high level, 10^{-2} M phosphate, in the treatment solution significantly phytotoxic by itself. Barley (Hordeum

vulgare L.) did not show the atrazine-phosphate combination effect evident in corn, peas, sorghum, and soybeans.

The enhanced phytotoxicity of atrazine in the presence of phosphate appeared to be related to a synergistic increase in respiration of corn, peas, sorghum, and soybean plants receiving the combination treatment. The atrazine induced inhibition of photosynthesis in these species was not synergistically enhanced by the additional presence of phosphate.

Although the trends were not statistically significant at the 5% level, at the 10^{-5} M atrazine level, increasing levels of phosphate tended to reduce total atrazine uptake during the treatment period. For corn, peas, and soybean this trend was reversed at the 10^{-4} M atrazine level.

The percent of chloroform-soluble metabolites, including the parent atrazine, increased with increasing atrazine and phosphate levels in corn and peas. If this effect were of sufficient magnitude it could explain enhanced phytotoxicity. The presence of phosphate with the atrazine treatment reduced the metabolism of atrazine to a methanol-insoluble residue and increased the

accumulation of hydroxylated metabolites in corn, peas, and soybeans. It is difficult to envision how this effect could explain the altered phytotoxicity unless the assumptions on the phytotoxicity of these metabolites are erroneous or this block in metabolism also affected the rate of conversion of atrazine to hydroxylated metabolites. There did not appear to be any effect of phosphate on atrazine metabolism by sorghum.

Atrazine did not affect the uptake or distribution of ^{32}P by corn.

The enhanced phytotoxicity of atrazine in the presence of high levels of phosphate can best be explained by an interaction on the rate of respiration. The increased respiration could be due to an interaction effect on the respiration apparatus or by an increase in the internal concentration of atrazine resulting from a slight increase in uptake and a slight decrease in metabolism of atrazine in the presence of high levels of phosphate. These alternatives are not mutually exclusive.

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INTRODUCTION

There have been several reports of interactions between herbicides and phosphate. One of the best documented is a synergistic reduction of plant growth by atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) and phosphate (1,2,9,32).

Atrazine is a widely used herbicide of great economic importance. It is often applied in a band at the same time as fertilizer application resulting in the close proximity between the herbicide and fairly high concentrations of phosphate fertilizer. Since it has been shown that atrazine and phosphate in the same medium may have a synergistic effect, it is possible that early seedling growth is reduced. If the reduction was limited to weed species, this could enhance weed control in the rows; but if the crop species was also affected, it would be advantageous to alter the fertilization or weed control program.

With the present emphasis on environmental quality, plans for recycling nutrients found in wastes have

been suggested. The high phosphate content of some of these wastes could result in situations where a phosphate-herbicide interaction could occur.

It is important to know the basis of this interaction in order to reduce a potential loss of herbicide selectivity or to exploit the interaction by applying less herbicide and reducing herbicide residue. It is the purpose of this work to study several systems which could be causal factors of the interaction.

LITERATURE REVIEW

s-Triazine Herbicide Selectivity

Most of the studies on the selectivity of s-triazine herbicides indicate that uptake and translocation of the herbicides have little effect on their selectivity. Moreover, the sensitivity of isolated chloroplasts to triazines measured as inhibition of the Hill reaction is found to be the same for tolerant and sensitive species (21). The ability of tolerant plant species to metabolize the toxic compounds to a non-toxic form appears to account for their tolerance.

Using the present knowledge about triazine action and metabolism, Shimabukuro and Swanson (28) have developed a model for selectivity. The site of triazine action is believed to be the chloroplast. s-Triazine metabolism is thought to occur in the cytoplasm. The chlorotriazines are very lipid soluble and can readily penetrate the lipid-rich chloroplasts of both tolerant and susceptible species. Atrazine present in the leaves

of treated plants will enter the chloroplasts and accumulate until an equilibrium exists with atrazine in the cytoplasm. Hydroxylated derivatives, however, are lipid insoluble so they are excluded from the site of herbicide action. Shimabukuro and Swanson (28) have shown that when leaf tissue of sorghum was treated with ^{14}C-atrazine, the percent of label in the cytoplasm increased with time as did oxygen evolution. In order to maintain the equilibrium of atrazine between chloroplasts and cytoplasm, atrazine was removed from the site of action as the compound was metabolized in the cytoplasm. Plants subsequently recovered photosynthetic ability.

It is not known if the hydroxylated atrazine derivatives have the molecular structure necessary for inhibiting the Hill reaction. But since these compounds may not reach the site of action this is of little consequence.

The literature is not without some controversy regarding s-triazine herbicide selectivity. Wheeler and Hamilton (34) exposed wheat, corn, and sorghum to atrazine in solution culture. Following prolonged treatment, they extracted the plant material and determined the amount of unaltered atrazine spectrophotometrically. Tolerant

species were found to accumulate leaf concentrations of herbicide which were comparable to those found in sensitive species at the point of acute toxicity. This suggested that more is involved in selectivity than simply detoxication. They offered the hypotheses that corn leaves may bind the herbicide at inactive sites within the corn leaf tissue or prevent a particular series of reactions which mediate the acute toxicity.

Even though the tolerant species did not show acute toxicity symptoms, a severe growth inhibition was observed. Preliminary observations indicated that photosynthesis was also inhibited in these species. If this is true, it might simply be an overloading of the detoxication mechanism. The "tolerant" species were not behaving as tolerant plants. They differed from the susceptible species only in the degree to which they showed acute toxicity symptoms.

Their data in (34) showed that wheat accumulated 9.0 ppm atrazine from a 1.0 ppm solution in 6 days. Corn and sorghum accumulated 4.0 ppm and 9.5 ppm respectively in 20 days from a 10.0 ppm solution. These data suggested active accumulation in wheat versus exclusion and/or metabolism by corn and sorghum. Their work did

not seem to exclude metabolism to non-toxic compounds as the primary basis for triazine selectivity.

s-Triazine-Herbicide Action

Extensive work has been reported in the literature on the action of the triazines (11,13). Most of the work has been done on simazine (2-chloro-4,6-bis (ethylamino)-s-triazine) and atrazine, the two compounds most widely used in weed control, although the action of other triazines has been studied (11,13).

Since the action of the s-triazines, particularly the chlorinated triazines, is generally the same, their action, selectivity and degradation can be considered as a group.

It is generally accepted that the s-triazines inhibit the photolysis of water depriving the chlorophyll system of electrons necessary for photosynthesis. This inhibition of the Hill reaction has been studied spectrophotometrically as change in absorbance with the reduction of potassium ferricyanide or Janus Green. The simazine concentration necessary for 50% inhibition (I_{50}) of this reaction on isolated chloroplasts was 7×10^{-7} M (11).

The chlorophyll concentration in the solution was much higher than the herbicide concentration. Moreland and Hill (21) using simazine found that at the I_{50}, the molar ratio of chlorophyll to herbicide was 5.3. This value was much higher than the ratios for most of the other inhibitors they tested. They suggest that simazine is fairly specific in its action. However since not a great deal is known about the photolysis of water, the exact molecular site of s-triazine action remains unknown.

Related to the loss of reducing power is the increase in fluorescence observed when photosynthesis inhibitors like the triazines are added to chlorophyll. The fluorescence results from the release of energy not captured by the carbon-reduction cycle. The pF_{50} agrees fairly well with the pI_{50} determined using the decreased rate of oxygen evolution.

The s-triazines have also been shown to inhibit non-cycic photophosphorylation, but the cycic photophosphorylation system was not inhibited (11). Uncoupling occurred only at triazine concentrations 100 times greater than that necessary to inhibit the Hill reaction.

A consequence of the inhibition of the Hill reaction is the inhibition of the reduction of NADP. The production of NADPH as well as ATP by the photosynthetic system is indispensable for the subsequent assimilation of CO_2. It is easy to measure this decrease in CO_2 assimilation by isolated chloroplasts or intact plants in the light. Labeling studies with $^{14}CO_2$ show that ^{14}C incorporation into sucrose in the presence of triazine herbicides is severely inhibited. Zweig et al. (37) found that 99% of photosynthetic CO_2-fixation could be blocked by atrazine in excised bean leaves. The triazines did not, however, inhibit dark fixation of CO_2. Zweig (36) found no effect on PEP-carboxylase-catalyzed CO_2-fixation. Zweig et al. (37) showed no effect of atrazine on the formation of aspartic or glutamic acids and other compounds associated with the TCA cycle. Comparing ^{14}C - labeling of these compounds, they found no difference between their dark control, atrazine in the dark, or atrazine in the light. Couch and Davis (8) obtained similar results.

In one case (18) simazine treatment of Norway Spruce (Picea abies) at 20 ppm stimulated photofixation of $^{14}CO_2$ but one would expect that the site of action for

this effect was not at the level of the photo reactions themselves.

Several studies indicate that the triazine herbicides do not affect carbohydrate metabolism, particularly the enzymes involved in starch synthesis. Ashton et al. (4) showed that cessation of growth of atrazine-treated algae (Chlorella vulgaris) could be overcome by the addition of exogenous glucose. This, plus the fact that glucose can prevent the atrazine-induced starch loss from plant cells, indicates that atrazine does not prevent the use of this exogenous energy source. Sucrose can also prevent starch loss (11). This is a fairly strong indication that atrazine acts at the level of monosaccharide synthesis without affecting subsequent carbohydrate metabolism.

Several studies have involved examination of atrazine-treated plants with an electron microscope. Changes in the fine structure of barnyardgrass chloroplasts have been observed by Hill et al. (16). Ultrastructural changes occurred before any macroscopic changes were evident. They could be seen after 2 hours of treatment with 2, 5, 10, and 20 ppm of atrazine. The degradation of the chloroplast began as swelling of the

inter-granal fret system followed by swelling and disruption of granal discs. In the more advanced stages of degradation, the membranes of the grana and chloroplast envelope were ruptured. They also observed a decrease in the number of starch grains present in the treated cells as treatment time exceeded four hours. Mitochondria appeared normal throughout the experiments, irrespective of concentration or length of treatment.

Ashton et al. (5,6) made similar observations using bean leaves but a 30-hour delay period was sometimes necessary before changes were evident.

The aforementioned studies were designed to show triazine herbicide effects on light systems. Ashton (3) using Kanota oats and red kidney beans studied the relationship between light and the toxicity symptoms of atrazine. Toxicity symptoms developed in the light but not in the dark and the degree of acute toxicity was shown to be a function of light intensity and quality. The action spectrum of atrazine injury corresponded to the absorption spectrum of chlorophyll, indicating that this pigment was involved in the expression of toxicity symptims. Ashton concluded that light was necessary for the expression of toxicity symptoms and suggested that atrazine

toxicity was not due to the compound *per se* but rather due to a secondary substance formed by some mechanism involving atrazine and light. Ashton (3) and Sweetser and Todd (30) came to similar conclusions for monuron (3-(*p*-chlorophenyl)-1,1-dimethylurea). Ashton *et al.* (5,6) suggested that a free radical might mediate the toxicity symptoms while Sweetser and Todd (30) thought there might be an accumulation of a toxic photosynthetic intermediate. No further evidence has been produced to support either hypothesis.

Several other effects of the triazines have been reported. Atrazine and simazine inhibited the growth of tobacco callus tissue in the dark, and in a medium containing sucrose (11). The I_{50} was 10^{-6} M, almost 20 times the concentration necessary to inhibit photosynthesis. Such results could explain why phytotoxicity is observed with some tissues in the dark. Ebert and Müller (11) suggest atrazine might affect plant hormones but the possibility that the action could be an enzymes involved in tissue growth should not be excluded.

Ries *et al.* (23) have studied the effect of simazine on protein and nitrogen metabolism. Rye was grown at 22°/17°C or 17°/12°C day/night temperatures. At

optimum concentrations of simazine, protein accumulation increased to levels as high as 79% above controls. There was no change in protein quality. Treated plants showed a slight increase in respiration with no change in respiratory quotient (RQ). The dry weight of treated plants was somewhat lower than for the control plants although the fresh weights were similar. Nitrate reductase activity increased in treated plants. The above observations held for plants grown on nitrate but not for plants receiving ammonia as the nitrogen source. The magnitude of the response decreased as the nitrate concentration approached the optimal nutritional level.

Pea plants grown in simazine contained 40% more protein in the seeds than non-treated plants. In similar studies, Tweedy and Ries (31) found an increase in the efficiency of nitrate utilization at low nitrate concentration and low temperature in treated versus non-treated plants.

Ebert and Van Assche (12) worked with soybean callus tissue grown on agar which contained kinetin. Atrazine increased growth measured as an increase in fresh weight over controls. The optimum range of concentration was 10^{-8} to 10^{-18} M.

s-Triazines have also been reported to affect the size of leaves and the diameter of the stems. Darker leaves as a result of higher chlorophyll and nitrogen have also been observed. In some cases, treatment can delay senescence. These observations led Ebert and Van Assche (12) to study the effect of atrazine on auxin. They reasoned that if atrazine inhibited peroxidase activity, IAA degradation would be inhibited. Enhancement of peroxidase activity on the other hand would result in lower IAA levels. They incubated decapitated oat coleoptiles for 9 hours in atrazine solutions of various concentrations, then transferred the coleoptiles into fresh treatment solutions which contained IAA-1-^{14}C. $^{14}CO_2$ was trapped over a 14-hour period and counted as a measure of enzyme activity. Peroxidase activity was increased at 10^{-6} to 10^{-8} M atrazine. Between 5×10^{-10} and 0.5×10^{-21} M atrazine, the range in which auxin-like effects were observed, enzyme activity was inhibited. While it is very difficult to imagine significant biological activity at herbicide concentrations less than one molecule per milliliter, it is possible that the effects might be real in the higher range of concentrations.

Good (13) studied several inhibitors of the Hill reaction in order to correlate activity with chemical structures. Although he felt a more diverse group of triazines needed to be studied before activity could be reliably predicted from molecular structure, he did make two tentative generalizations. First, chlorine at the 2-position resulted in a more active herbicide than a methoxy group substitution. Secondly, the presence of two imino hydrogens gave greater phytoxicity than the presence of only one, which in turn resulted in greater activity than none at all. Moreland and Hill (21) obtained similar results. They concluded that the chlorine and imino hydrogens were important for spacial orientation. They found no apparent correlation between water solubility and herbicidal activity. Solubilities were 5, 8.6, 10, and 3200 ppm in water for simazine, propazine (2-chloro-4,6-bis(isopropylamino-s-triazine), chlorazine (2-chloro-4,6-bis(diethylamino)-s-triazine), and methoxysimazine (2-methoxy-4,6-bis(isopropylamino)-s-triazine) respectively. The order of activity is simazine > propazine > methoxysimazine > chlorazine.

Metabolism of s-Triazine Herbicides

The most extensive studies on the metabolism of triazine herbicides have been done on atrazine and simazine. The first metabolites to be isolated and identified were the hydroxylated derivatives of simazine and atrazine, 2-hydroxy-4,6-bis(ethylamino)-s-triazine and 2-hydroxy-4-ethylamino-6-isopropylamino-s-triazine, respectively (20). This conversion was found to be non-enzymatically catalyzed by the cyclic hydroxamate, 2,4-dihydroxy-3-keto-7-methoxy-1,4-benzoxazine (benzoxazinone).

Montgomery and Freed (20) cited evidence of further breakdown of the triazine molecule. They reported that $^{14}CO_2$ was given off by corn plants treated with ^{14}C-atrazine or ^{14}C-simazine although they could not show this with labeled prometryne (2-methylmercapto-4,6-bis (isopropylamino)-s-triazine) or propazine. They proposed a metabolism scheme whereby hydroxylation at the 2-position was followed by cleavage of the ring to form $^{14}CO_2$ and a substituted biguanide. The biguanide was then thought to be hydrolized to form a biuret or a substituted guanidine and a substituted urea.

Hamilton (14) studied the tolerance of plant species to the chloro-*s*-triazines in relation to their benzoxazinone content. He found that the ability of excised roots to metabolize triazines to their hyroxy derivatives was directly related to their benzoxazinone content. Of the species tested rye, corn, and wheat contained benzoxazinone but only corn was tolerant. Sorghum, another tolerant species, contained no benzoxazinone. Clearly, non-enzymatic conversion of chloro-*s*-triazines to their hydroxy derivatives could not completely explain selectivity.

Following a report that the soil fungus, *Aspergillus fumigatus*, Fres, was able to dealkylate simazine, Shimabukuro *et al*. (27) isolated and identified metabolites of atrazine from mature pea plants, *Pisum sativum*. Employing infra-red spectroscopy and thin layer chromatography they identified the compound 2-chloro-4-amino-6-isopropylamino-*s*-triazine (Compound I) and designated 2-chloro-4-amino-6-ethylamino-*s*-triazine as Compound II. Another metabolite in addition to hydroxyatrazine was detected but was not identified.

In a subsequent series of papers Shimabukuro and his co-workers (17,25,26,27,28,29) further elucidated

atrazine metabolism. Compound I was found to be a major atrazine metabolite in both the roots and shoots of pea. Only a small amount of atrazine was metabolized to water-soluble and methanol-insoluble compounds. Hydroxyatrazine was detected after 48 hours indicating that very little metabolism beyond dealkylation occurred in this period of time.

The herbicidal action of Compound I was compared to atrazine. No difference at 10^{-7} and 10^{-6} M concentrations was observed but at 10^{-5} M, Compound I was less toxic as measured by the reduction in root and shoot dry weight. The I_{50} for Compound I, measured as inhibition of the Hill reaction on isolated pea chloroplasts was 4.6 x 10^{-5} M or 23 times greater than the I_{50} for atrazine at 2.0 x 10^{-6} M (28). Shimabukuro concluded that accumulation of this less toxic atrazine derivative was responsible for the intermediate sensitivity of pea plants to atrazine.

Shimabukuro (25) next studied atrazine metabolism in corn, sorghum, pea, wheat, and soybean. He found that all of these could metabolize atrazine by N-dealkylation. Corn and wheat, because they contained benzoxazinone could also convert atrazine to hydroxyatrazine. Subsequent

metabolism resulted in the formation of more polar compounds and other compounds found in the methanol-insoluble residue. Contrary to the results of Montgomery and Freed (20) there was no evidence for cleavage of the triazine ring. Susceptible species contained more unaltered atrazine than the tolerant ones. In sorghum, a tolerant species, atrazine content decreased with time. The insoluble residue increased with time at the expense of atrazine and unidentified water soluble compounds.

Shimabukuro (26) compared the metabolism of atrazine in corn and sorghum. Sorghum, as shown before (25), could metabolize atrazine by N-dealkylation but in this experiment, Compound II was also isolated. Corn formed both Compounds I and II in addition to hydroxyatrazine. Two additional water-soluble metabolites were also identified. These were 2-hydroxy-4-amino-6-isopropylamino-s-triazine (Hydroxycompound I) and 2-hydroxy-4-amino-6-ethylamino-s-triazine (Hydroxycompound II). These could be formed either by N-dealkylation of hydroxyatrazine or by hydroxylation of Compound I or II.

Shimabukuro and co-workers (17) have subsequently identified a new pathway for atrazine metabolism in

sorghum involving the formation of S-(4-ethylamino-6-isopropylamino-2-s-triazino)glutathion and γ-L-glutamyl-S-(4-ethylamino-6-isopropylamino-2-s-triazino)-L-cysteine. Two additional water-soluble metabolites were formed after treatment for extended periods of time but they have not yet been identified. The most complete scheme presently available for atrazine metabolism is shown in Fig. 1 (17).

Montgomery et al. (19) have shown that hydroxysimazine is metabolized to Hydroxycompound II.

s-Triazine Herbicide-Phosphate Interactions

Several workers have observed that high levels of phosphate caused increased phytotoxicity of several herbicides. Upchurch et al. (32) studied 12 herbicides and found statistically significant herbicide-phosphate interaction for diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl urea], amitrole (3-amino-s-triazole), CDAA (N,N-diallyl-2-chloroacetamide) and simazine.

Adams (1) suggested that the herbicide simazine reduced the amount of phosphorus required to cause salt toxicity. Dhillon et al. (9) found that simazine at 20 ppm

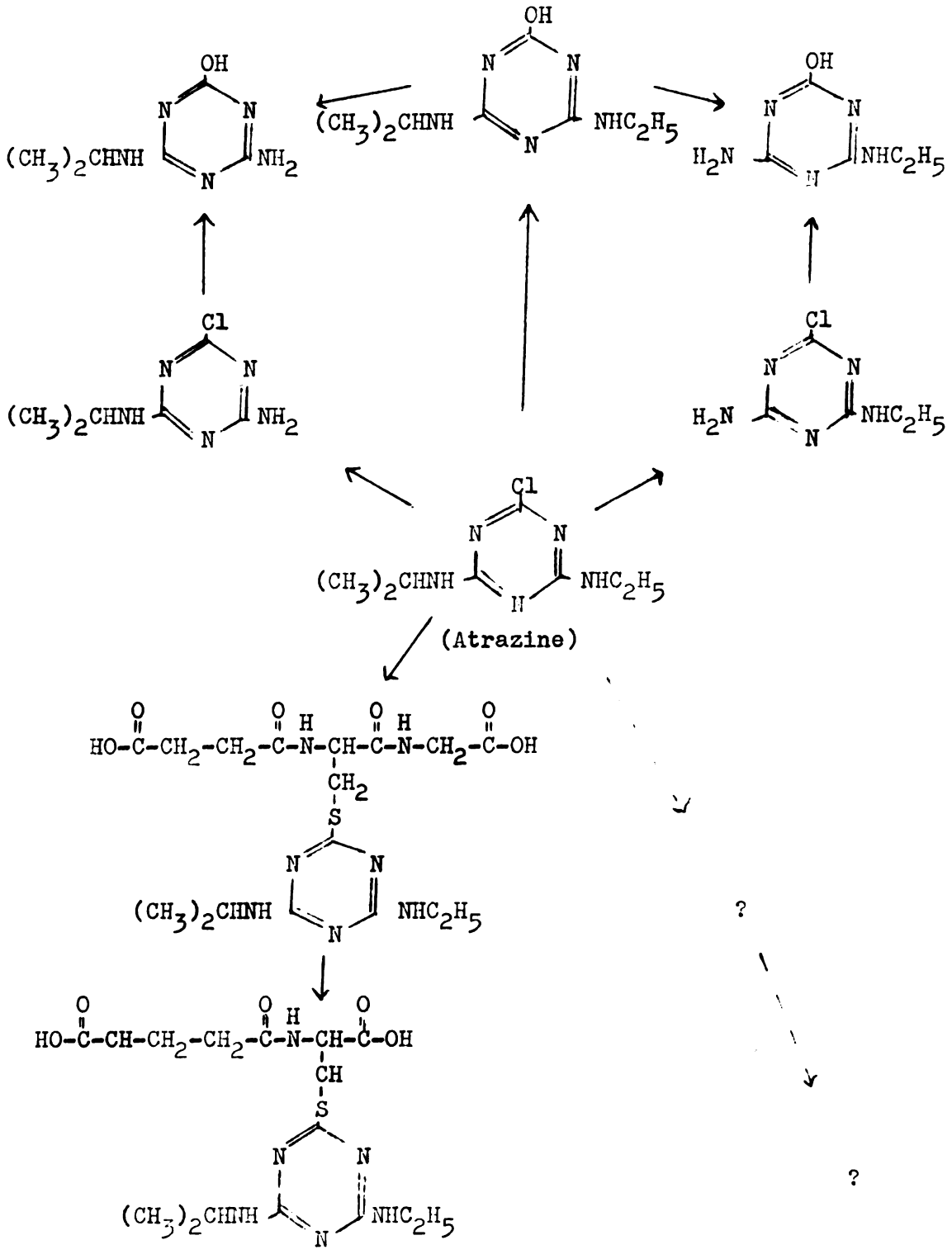

Fig. 1.--Atrazine metabolism in higher plants as postulated by Lamoureux et al. (17).

decreased the rate of phosphorus uptake and accumulation in roots and subsequently increased its translocation from roots to stem and needles of red pine seedlings.

More recently Adams (2) studied the effects of phosphorus and atrazine treatments on the mineral composition of soybeans. He concluded that phosphorus appeared to increase the sensitivity of soybeans to atrazine.

Doll et al. (10) studied the influence of herbicide and phosphate combinations on the root absorption of amiben and atrazine. They concluded that the herbicide-phosphate interaction they observed was not due to increased uptake of either of the herbicides in the presence of phosphate by roots of corn, soybean, squash, or pigweed.

Penner (22) found that inhibition of phytase activity by amiben and atrazine was enhanced by high but not phytotoxic levels of phosphate in the culture medium.

Herbicide-phosphate interactions could result from any one of several possibilities. As Adams first suggested (1) the herbicide could make the plant more sensitive to phosphate salt toxicity. Conversely, as he has later concluded, phosphate could make the plant more sensitive to herbicide injury. The possibility that the

herbicide affects the uptake of phosphorus or vice versa has already been investigated and does not appear to explain the interaction. Effects on the status of other nutrients have also been studied and there seems to be little need to pursue that alternative further.

If phosphate is affecting the herbicide phytotoxicity it could do so either by acting at the site of herbicide action in the plant or by influencing the fate of the herbicide itself in the plant. This latter possibility could be particularly significant in those cases where the basis of selectivity is detoxication by tolerant plant species. The examination of these two possibilities is consistent with the main objective of the thesis, namely to determine the basis for the atrazine-phosphate interaction.

MATERIALS AND METHODS

Culture Technique and Growth Measurements

Corn (*Zea mays* L., var. 400-F26), soybeans (*Glycine max* Merril), va. Hark and barley (*Hordeum vulgare* L. var. Larker) were planted, ten seeds per cup, in washed quartz sand. The sand was placed in 6 oz. styrafoam hot cups with drainage out the bottom. These cups were in turn placed within 10 oz. wax cups with drain holes cut in the side to maintain a constant level of solution when filled to run-out. Treatment solutions were added every two days for the first several days and daily when necessitated by higher rates of transpiration.

All plants in the study were grown in controlled environment chambers. Light intensity at the top of the cups was 2000 foot candles from mixed fluorescent and incandescent sources. The photoperiod was 16 hours of light and 8 hours of darkness. The temperature was held constant at 30°C for corn and soybeans and 20°C for barley.

The experimental design in the study determining atrazine-phosphate interaction on corn, soybean, and barley growth was a 4 x 4 factorial. There were two replications and the experiment was repeated a second time. Atrazine concentrations were 10^{-4}, 10^{-5}, 10^{-6} and 0 M. Phosphate as an equimolar mixture of mono- and dibasic potassium phosphate was added at 10^{-2}, 10^{-3}, 10^{-4}, and 0 M. Solutions containing phosphate were naturally buffered at a pH of 6.8. The other solutions were adjusted to a pH of 6.8 to 7.0. No additional nutrients were added to the solutions.

Plants were harvested after 7, 8, and 9 days for corn, barley, and soybean, respectively. At this age, growth inhibition by several treatments was evident by visual observation. The plants were removed from their cups and the sand was washed from the roots. Roots and shoots were separated and the remains of the seed or, in the case of soybean, the cotyledons, were removed and discarded. Total shoot height was measured and the plants counted. Root and shoot were oven dried separately and weighed.

Peas, Pisum sativum L., var Progress No. 9 and sorghum, Sorghum vulgare L., var. Forage Sorghum Hybrid,

were planted in a 3 x 3 factorial design and grown at 20 and 30°C, respectively. The lowest concentrations of atrazine and phosphate, i.e., 10^{-6} M and 10^{-4} M respectively, were eliminated. A modified Hoagland's No. 1 nutrient solution without phosphate was added to all solutions. Pea and sorghum plants were harvested at 14 and 8 days respectively. There was a total of four replications of peas and five of sorghum.

Measurement of Respiration and Photosynthesis

Atrazine-tolerant corn and sorghum, intermediately susceptible peas, and susceptible soybeans plants grown as previously described were used in this study. Two replications of each species were treated in a 3 x 3 factorial design. Concentrations of atrazine, phosphate, and nutrients were the same as for peas and sorghum. In addition, ^{14}C-atrazine was added to those solutions which contained unlabeled atrazine. The solutions contained 5 μc of ^{14}C-atrazine per liter. A small aliquot of each treatment solution was counted so all of the final data could be corrected for concentration differences.

Plants were grown 7, 8, 10, and 14 days for corn, soybeans, sorghum, and peas respectively. On the day of harvest, respiration and photosynthesis was determined for the plants in each cup using a Beckman Infra-red Analyzer Model IR 215.

The experimental apparatus consisted of an open-flow system which was operated at a constant flow rate (500 ml/min) measured at the exit port of the analyzer. Air from a compressed air tank passed through tygon tubing into the controlled environment chamber where it entered the plant treatment chamber (Fig. 2). This chamber was made from clear plastic pipe, sealed on both ends. The pipe was cut near the base and the base fitted with a sleeve. The union between the two parts of the chamber was sealed with masking tape.

After passing over the plant, air moved through tygon tubing to the outside of the growth chamber. It was dried by passing it through tubes of $CaSO_4$ dessicant which was changed as necessary. The dried air was then analyzed for CO_2 content.

The apparatus was calibrated by zeroing with nitrogen. Half scale deflection was adjusted to compressed air passed directly through the analyzer. The difference

Fig. 2.--Plastic plant chamber used in CO_2 analysis.

between 0 and half-scale deflection was assumed to be 0.3% CO_2. Distances of deflection on the chart were determined as ppm per unit and the data was calculated as μg CO_2 per min per unit area or weight.

After respiration and photosynthetic rates had been determined, plants were removed from the growth chamber and cut off at the crown. The leaves were traced on notebook paper and total leaf area was determined using a planimeter. After the plants were measured they were frozen on dry ice and placed in a freezer until all the pots had been harvested. The plant material was freeze-dried, weighed, and held for extraction of ^{14}C-atrazine and its metabolites.

Extraction of ^{14}C-Atrazine and Its Metabolites

The extraction procedure for atrazine and its metabolites was essentially that used by Shimabukuro (24).

The freeze-dried plant material was homogenized with 30 ml of 95% methanol. The extract was filtered and the residue re-extracted twice with an additional 20 ml of methanol. The filtrate was placed in a hot water bath

at 70°C and shaken gently for 15 minutes. A portion of the residue was folded in a preweighed piece of black paper for combustion and assay of the methanol-insoluble ^{14}C-atrazine metabolites.

The methanol extract was evaporated under vacuum in a flash evaporator leaving the aqueous fraction. This was washed from the boiling flask and centrifuged for 15 min at 13,300 x g. The pellet was washed twice by resuspending it in water and then recentrifuging. A 0.5 ml aliquot of the supernatant fluid was removed for counting.

The aqueous fraction was then washed five times with equal volumes of chloroform. The chloroform soluble fraction which includes unaltered atrazine and Compounds I and II was evaporated to dryness under vacuum. The residue was removed from the boiling flask with 10 ml of absolute methanol and placed in a vial for the determination of radioactivity.

The samples now dissolved in methanol were evaporated to dryness under nitrogen. The residue was redissolved in 1 ml of absolute ethanol. A 0.5 ml aliquot was removed for the determination of radioactivity. The remaining sample was saved for spotting thin-layer chromatograph (TLC) plates.

Control extractions were done using weighed amounts of untreated plant material. A known amount of ^{14}C-atrazine was added to this material when it was first homogenized. Two samples of each species were then carried through the aforementioned extraction procedure. Two additional samples of corn were extracted as outlined by Shimabukuro (25). The only additional step involved boiling the aqueous extract for five minutes before the chloroform wash to inactivate the benzoxazinone.

Separation and Identification of ^{14}C-Atrazine and Its Metabolites

Unaltered atrazine and its chloroform soluble metabolites were separated by TLC on 250 μ thick silica gel H plates developed in benzene:acetic acid (50:4, v/v). Six samples and a pure ^{14}C-atrazine standard were run on each plate. After they had been developed, the plates were divided into zones and the silica gel scraped into vials for the determination of radioactivity. The two plates with extracts from corn were scraped in 10 zones of 1.5 cm each. All other plates were scraped into 13 zones including the origin and 7 1-cm zones from the

lower half of the plate and five 1.5-cm zones from the remaining upper portion.

Positive identification of the metabolite remaining at the origin was determined by multiple thin-layer chromotography. Three samples of corn extract containing almost solely metabolites that remained at the origin were spotted and developed in the same solvent system as above. The origin was scraped and the gel washed with ethanol to remove the radioactivity. The resulting solution was dried under nitrogen and redissolved in 0.1 ml ethanol, half of which was re-spotted on a second plate which was then developed in benzene:acetic acid:water, 50:50:3 (v/v/v). Hydroxyatrazine ran at R_f 0.27 in this system. Plates were scraped into 1 cm zones, and placed in vials for counting.

Quantitative determination of methanol-insoluble atrazine metabolites were made by the Schoeninger combustion method of Wang and Willis (33). The papers containing the plant residue were dried and weighed and placed in sealed vacuum flasks. The flasks were evacuated and refilled three times with pure oxygen to atmospheric pressure. The samples were then ignited with a beam of infrared light.

Twenty ml of ethanol-ethanolamine (2:1) was injected into the flask through a serum cap in the top. After stirring, 20 minutes were allowed for this mixture to absorb the $^{14}CO_2$ in the flask. A 5 ml aliquot was removed for counting. Fifteen ml of scintillation solution containing 5 grams PPO and 0.3 grams of dimethyl POPOP per liter of toluene were added to each vial.

The scrapings from the TLC plates were first dissolved in 0.5 ml of absolute ethanol before 15 ml of the scintillation solution was added. This scintillation solution containing 5 grams PPO and 0.5 grams POPOP per liter of 30% absolute ethanol in toluene.

Two different Packard Tri-carb counters were used. Samples were counted either at 14% gain or 24% gain on channel II. Window settings were 50-1000. Gain on channel I was 4%. All samples were corrected for quenching using the channels ratio method.

Assay of Atrazine Effect on ^{32}P Uptake by Corn

To check the effect of atrazine on phosphate uptake and distribution an experiment was designed, adding

^{32}P to the culture solutions. Corn plants were grown as previously described in a 2 x 2 factorial with two replications. The concentrations of atrazine and phosphate were 0 and 10^{-4} M and 0 and 10^{-2} M respectively. Culture solutions at this point contained only unlabeled phosphorus. When the plants were seven days old, they were removed from the cups and the sand was washed off the roots. Five representative plants were selected for ^{32}P treatment. These were placed into 100 ml of treatment solution in wax cups to which had been added 0.1 mc ^{32}P as KH_2PO_4. The plants were placed back into the controlled environment chamber at 30°C for 6 hours. Solutions were aerated as shown in Fig. 3.

After the treatment period, the plants were removed from the solutions and the roots rinsed four times with distilled water. The plants were frozen on dry ice and then freeze dried and prepared for autoradiography.

The plant material was found to contain large amounts of ^{32}P and several exposure times were necessary to obtain good pictures. Final exposure times were 16 hours for treatments containing 10^{-2} M phosphate in solution and 40 minutes for the treatments without phosphorus.

Fig. 3.--^{32}P treatment of corn in aerated solution culture for uptake and distribution study.

The difference in time was due to a dilution of ^{32}P when unlabeled phosphate was present.

Labeled phosphate was determined quantitatively using the methods of White and Ellis (35). Treatments were made in duplicate for autoradiography and two samples were taken from each of these making four replications. Two or three plant shoots were removed from the blotter paper and ground in a Wiley mill (40 mesh screen). The plant material was collected in preweighed crucibles which were then reweighed. The material was placed in a muffle oven for 6 hours at 400°C. The material was allowed to cool and 10 ml of 1 N HNO_3 was added to each crucible. The acid was evaporated slowly on a hot plate and the crucibles heated again for 10 minutes at 400°C. The residue was redissolved in 10 ml 2 N HCl and these solutions transferred to scintillation vials for counting without additional scintillators using Cerenkov radiation.

A Packard Tri-carb Scintillation Spectrometer was used for counting the samples. The gain setting was 50%. Quenching was assumed to be uniform so data was calculated as counts per minute per unit weight of plant material.

RESULTS AND DISCUSSION

Atrazine-Phosphate Effects on Plant Growth

Typical injury symptoms of atrazine-phosphate combinations on corn are shown in Fig. 4. Plants in the combination treatments were shorter than other plants and often were malformed. They were usually lighter in color showing typical atrazine injury.

Average values for plant height, shoot weight, and root weight on a per plant basis are given in Tables 1 through 5. Duncan's Multiple Range Test was used to compare means when an analysis of variance showed them to be significant at the 5% level.

Two basic patterns can be seen in the data. In the species susceptible to atrazine injury, soybeans and barley (Tables 1 and 2), injury was due to atrazine with no effect of phosphate concentration on barley growth and only small effects on soybean growth. For this reason barley was not used after the initial growth experiment.

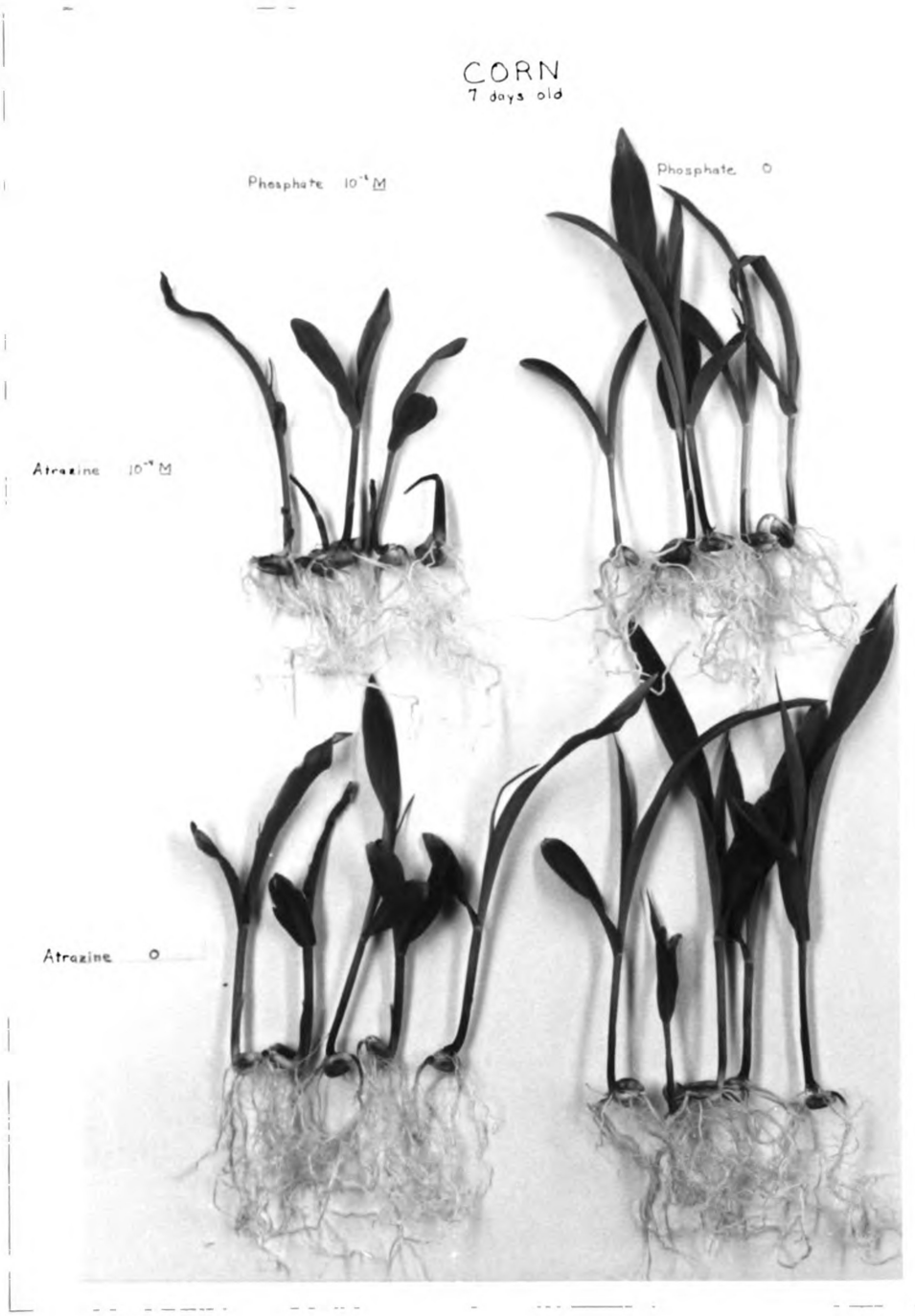

Fig. 4.--Atrazine-phosphate combination effects on the growth of 7-day-old corn.

In soybeans, the combined inhibition by atrazine and phosphate tended to be more than additive for plant height and shoot weight. There was a significant synergistic height reduction for atrazine and phosphate at 10^{-6} M and 10^{-2} M respectively and a synergistic interaction approaching significance at 10^{-5} M atrazine and 10^{-2} M phosphate.

Using the same comparisons as above, sorghum, a tolerant plant, was shown to behave similarly to soybeans and barley, but to a lesser degree (Table 3). Atrazine at either 10^{-4} or 10^{-5} M significantly reduced plant height, shoot and root weight to levels below that of the control. Plants receiving phosphate without atrazine were different from the control only in reduced root weight. Except for root weight the values of the combined inhibition effects were more than additive at the high atrazine concentration.

Peas follow a second pattern in which phosphate injury is readily apparent (Table 4). Atrazine alone at the high concentration was not significantly different from the control. The combination treatment was not significantly more toxic than the phosphate treatment alone,

however, the injury was more than additive for plant height. The observed decrease in root weight for the combination treatment was approximately additive.

Neither atrazine nor phosphate alone caused a significant decrease in corn height compared with the controls (Table 5). Combined inhibition with phosphate at 10^{-2} M and atrazine at 10^{-4} or 10^{-5} M appeared to be more than additive although these combination treatments were not significantly different from the phosphate treatment alone. Shoot weights showed no significant differences between treatments. Root weight, as in sorghum and peas, was more sensitive to atrazine than shoot weight and the combination treatment showed only additive effects of the atrazine and phosphate treatments.

The results of these preliminary experiments show that an atrazine-phosphate interaction does occur and can be measured at least for corn, sorghum, peas, and soybean. Consequently these species were used in subsequent experiments to further study this interaction.

TABLE 1.--Atrazine-phosphate combination effects on soybean growth.[1]

Atrazine concentration	Phosphate concentration	Height mm	Shoot weight mg	Root weight mg
10^{-4} M	10^{-2} M	38 a[2]	17 a	9 a
10^{-4} M	10^{-3} M	50 ab	21 ab	20 abc
10^{-4} M	10^{-4} M	44 ab	20 ab	19 abc
10^{-4} M	0	54 abc	25 abc	20 abc
10^{-5} M	10^{-2} M	61 bc	27 abcd	12 ab
10^{-5} M	10^{-3} M	97 def	32 bcd	22 abcd
10^{-5} M	10^{-4} M	86 d	29 abcd	24 bcde
10^{-5} M	0	89 def	28 abcd	22 abcd
10^{-6} M	10^{-2} M	60 abc	32 bcd	25 bcde
10^{-6} M	10^{-3} M	111 f	39 def	28 cdef
10^{-6} M	10^{-4} M	96 ef	34 cde	30 cdefg
10^{-6} M	0	99 def	33 cde	36 defg
0	10^{-2} M	75 cd	47 f	37 efg
0	10^{-3} M	94 def	45 ef	36 defg
0	10^{-4} M	88 def	34 de	43 g
0	0	91 def	38 def	42 fg

[1]Values are the mean of 4 replications with 10 plants per replication.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 2.--Atrazine-phosphate combination effects on barley growth.[1]

Atrazine concentration	Phosphate concentration	Height mm	Shoot weight mg	Root weight mg
10^{-4} M	10^{-2} M	80 ab[2]	8 ab	2 a
10^{-4} M	10^{-3} M	73 a	7 ab	2 a
10^{-4} M	10^{-4} M	66 a	6 a	2 a
10^{-4} M	0	83 abcd	8 ab	2 a
10^{-5} M	10^{-2} M	100 bcd	9 b	4 b
10^{-5} M	10^{-3} M	102 cd	9 b	4 b
10^{-5} M	10^{-4} M	99 bcd	8 ab	4 b
10^{-5} M	0	99 bcd	8 ab	4 b
10^{-6} M	10^{-2} M	103 cd	9 b	4 b
10^{-6} M	10^{-3} M	103 cd	9 b	4 b
10^{-6} M	10^{-4} M	104 cd	9 b	5 b
10^{-6} M	0	103 cd	8 ab	5 b
0	10^{-2} M	105 cd	14 c	9 d
0	10^{-3} M	118 d	16 d	8 cd
0	10^{-4} M	110 d	13 c	7 c
0	0	107 d	12 c	8 cd

[1]Values are the mean of 4 replications with 10 plants per replication.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 3.--Atrazine-phosphate combination effects on sorghum growth.[1]

Atrazine concentration	Phosphate concentration	Height mm	Shoot weight mg	Root weight mg
10^{-4} M	10^{-2} M	75 a[2]	9 a	3 a
10^{-4} M	10^{-3} M	90 ab	10 a	4 a
10^{-4} M	0	100 ab	11 a	4 a
10^{-5} M	10^{-2} M	100 ab	13 a	4 a
10^{-5} M	10^{-3} M	119 bc	13 a	4 a
10^{-5} M	0	104 ab	12 a	5 a
0	10^{-2} M	124 bc	19 b	8 b
0	10^{-3} M	160 d	21 b	8 b
0	0	138 cd	18 b	11 c

[1]Values are the mean of 4 replications with 10 plants per replication.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 4.--Atrazine-phosphate combination effects on pea growth.[1]

Atrazine concentration	Phosphate concentration	Height mm	Shoot weight mg	Root weight mg
10^{-4} M	10^{-3} M	25 a[2]	23 a	21 a
10^{-4} M	10^{-3} M	42 bcd	42 abc	27 a
10^{-4} M	0	45 bcd	45 bcd	33 ab
10^{-5} M	10^{-2} M	38 bc	40 abc	32 ab
10^{-5} M	10^{-3} M	42 bcd	47 bcd	30 ab
10^{-5} M	0	45 bcd	44 bcd	28 a
0	10^{-2} M	35 ab	36 ab	27 a
0	10^{-3} M	52 d	59 cd	49 c
0	0	49 cd	64 d	43 bc

[1]Values are the mean of 4 replications with 10 plants per replication.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 5.--Atrazine-phosphate combination effects on corn growth.[1]

Atrazine concentration	Phosphate concentration	Height mm	Shoot weight mg	Root weight mg
10^{-4} M	10^{-2} M	69 a[2]	54	32 ab
10^{-4} M	10^{-3} M	127 cde	69	32 ab
10^{-4} M	10^{-4} M	98 abc	61	27 a
10^{-4} M	0	120 bcde	69	34 abc
10^{-5} M	10^{-2} M	79 ab	53	33 abc
10^{-5} M	10^{-3} M	149 e	81	47 bcd
10^{-5} M	10^{-4} M	139 cde	74	44 bcd
10^{-5} M	0	137 cde	67	46 bcd
10^{-6} M	10^{-2} M	103 abcd	77	47 bcd
10^{-6} M	10^{-3} M	138 cde	76	52 d
10^{-6} M	10^{-4} M	145 de	73	48 cd
10^{-6} M	0	132 cde	70	38 abcd
0	10^{-2} M	102 abcd	71	45 bcd
0	10^{-3} M	146 de	82	48 cd
0	10^{-4} M	143 de	77	53 d
0	0	145 de	74	50 d

[1]Values are the mean of 4 replications with 10 plants per replication.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

Atrazine-Phosphate Combination Effects on Plant Respiration and Photosynthesis

Plant height, shoot weight, and leaf area were three measurements available for evaluating the atrazine-phosphate combination effects on plant growth in this study. Since there were only two replications of these treatments, it was more difficult to obtain statistically significant results. The statistically significant results of the preliminary experiments reported here and in past research (1,2,9,32) are considered to be evidence enough that an interaction does occur.

The growth response of plants used for the photosynthesis and respiration studies supplied with phosphate, atrazine or the combination of the two is reported in Tables 6 to 9. The phosphate level has a significant effect on leaf area, plant height, and dry weight of corn plants. At 10^{-5} M, it promoted growth as measured by leaf area and plant height, whereas these values were significantly lower than the controls at the 10^{-2} M phosphate level (Table 6). The first response suggests growth stimulation of phosphate-starved plants, the second phosphate toxicity. This phosphate stimulation

occurred at all atrazine levels for area, height, and weight. There was no atrazine toxicity to plant growth at 10^{-5} M and only limited toxicity at 10^{-4} M showing up as a height reduction. Although the differences were not significant at the 5% level, the combination treatment of high levels of both atrazine and phosphate resulted in a more than additive inhibition.

Respiration of corn plants was not affected by phosphate alone. Atrazine only slightly inhibited respiration at the 10^{-2} M level. The combination treatment almost doubled the respiration rate above its expected value. This depletion of energy reserves would be expected to cause a reduction in plant growth.

Net photosynthesis was reduced by both atrazine and phosphate at their highest levels. In combination at those concentrations, they tend to have less than additive effects. The differences between means were not significant for total photosynthesis. There, too, atrazine at high concentration tended to reduce photosynthesis but the reduction due to the combination treatment was less than expected.

Only the 10^{-4} M concentration of atrazine showed a significant inhibitory effect on the dry weight of peas

grown for respiration and photosynthesis studies (Table 7). The leaf area and plant height were not affected by atrazine. Phosphate by itself tended to reduce leaf area and plant weight but not significantly. Plant height was uneffected.

Combination treatments showed only additive inhibition at the highest concentrations of atrazine and phosphate. The significant interaction at 10^{-5} M atrazine, 10^{-2} M phosphate did not show up in the preliminary experiment. The present result is partly due to the death of one replication.

The inhibitory effect of the combination on growth can be partially explained in peas on the basis of a significant synergistic promotion of respiration (Table 7). The observed respiration rate was approximately two to four times that which would have been expected if the effects were additive. There was no significant promotion of respiration by phosphate alone while atrazine alone reduced rather than increased respiration.

The rate of photosynthesis in treated peas indicates that atrazine was very inhibitory and that phosphate level had very little if any effect (Table 7).

Neither phosphate nor atrazine alone caused a significant reduction in sorghum growth (Table 8). The combination treatment using high atrazine and phosphate levels was more toxic than phosphate at 10^{-2} M by itself as indicated by plant height, area, and weight, and more toxic than the high atrazine level treatment as indicated by plant height. In all three cases, the growth in the above combination treatment was significantly less than the control. The combined inhibition by atrazine and phosphate at the high concentrations tended to be more than additive for area and weight and were significantly more than additive for height.

With sorghum, there was no significant effect of phosphate or atrazine level on respiration rate (Table 8). Plants receiving the combination treatment at high concentrations respired at a rate almost three times that of the control, a more than additive effect.

Net photosynthesis was reduced whenever atrazine was present in the nutrient solution (Table 8). There was also a reduction in net photosynthesis as the phosphate concentration increased. The same was true for total photosynthesis where atrazine level had a significant

inhibitory effect. The phosphate level did not affect total photosynthesis.

In this part of the experiment, soybeans showed less injury from atrazine than in Table 1. There was a promotion of plant growth by phosphate at 10^{-3} M for height and area and at 10^{-2} M for weight (Table 9). However, high phosphate levels tend to reduce growth. Increasing atrazine level significantly decreased plant height, weight, and leaf area. The growth inhibition by high atrazine and phosphate combination tended to be more than additive.

Atrazine at 10^{-4} M in the nutrient solution significantly increased soybean respiration (Table 9). At the high atrazine level there was no inhibitory interaction with phosphate. The respiration rate in the presence of both 10^{-5} M atrazine and 10^{-2} M phosphate in the nutrient solutions was twice that of the atrazine effect alone and 3 to 4 times that of the phosphate effect alone.

The low light intensity present in this experiment precluded significant photosynthetic rates by soybeans, consequently combination effects on photosynthesis could not be measured (Table 9).

TABLE 6.--Atrazine-phosphate combination effects on respiration and photosynthesis of corn.[1]

Atrazine concentration	Phosphate concentration	Leaf surface area cm^2	Plant weight mg	Plant height mm	Respiration $\mu g\ CO_2/min/g$	Net photosynthesis $\mu g\ CO_2/min/cm^2$	Total photosynthesis $\mu g\ CO_2/min/cm^2$
10^{-4} M	10^{-2} M	6 a[2]	27 a	78 a	632	0.00 a	1.82
10^{-4} M	10^{-3} M	16 bcd	50 bc	129 bc	388	0.40 abc	1.61
10^{-4} M	0	15 bcd	46 abc	127 bc	337	0.69 bcd	1.81
10^{-5} M	10^{-2} M	12 abc	43 ab	114 b	495	0.87 cd	2.66
10^{-5} M	10^{-3} M	20 cde	55 bc	149 de	452	1.09 d	2.36
10^{-5} M	0	11 bcd	48 bc	137 cd	495	0.89 cd	2.30
0	10^{-2} M	10 ab	41 ab	95 a	471	0.21 ab	1.55
0	10^{-3} M	27 e	66 c	167 e	402	1.04 d	2.05
0	0	22 de	55 bc	148 de	454	1.23 d	2.40
Average effect of levels							
10^{-4} M	-	12 b	41	111 b	452	0.36 a	1.74
10^{-5} M	-	16 ab	48	133 a	481	0.95 b	2.44
0	-	20 a	54	137 a	442	0.83 b	2.00
-	10^{-2} M	9 b	37 b	96 a	533	0.36 a	2.01
-	10^{-3} M	21 a	57 a	148 b	414	0.84 b	2.01
-	0	18 a	50 a	137 c	429	0.94 b	2.17

[1]Values are the mean of two replications which contained seedling germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 7.--Atrazine-phosphate combination effects on respiration and photosynthesis of peas.[1]

Atrazine concentration	Phosphate concentration	Leaf surface area cm^2	Plant weight mg	Plant height mm	Respiration µg CO_2/min/g	Net photosynthesis µg CO_2/min/cm^2	Total photosynthesis µg CO_2/min/cm^2
10^{-4} M	10^{-2} M	11 ab[2]	50 bc	34 abc	498 b	0.00	0.02 a
10^{-4} M	10^{-3} M	12 ab	42 ab	30 ab	572 b	0.00	0.08 a
10^{-4} M	0	18 ab	51 bc	34 abc	284 ab	0.00	0.03 a
10^{-5} M	10^{-2} M	2 a	29 a	28 a	917 c	0.00	0.00 a
10^{-5} M	10^{-3} M	15 ab	49 bc	32 abc	434 ab	0.66	0.11 a
10^{-5} M	0	26 b	64 cd	44 bc	198 a	0.00	0.01 a
0	10^{-2} M	18 ab	67 cd	45 bc	337 ab	0.20	1.59 b
0	10^{-3} M	21 b	71 d	46 c	346 ab	0.24	1.42 b
0	0	23 b	72 d	46 bc	308 ab	0.24	1.22 b
Average effect of levels							
10^{-4} M	-	14	48 a	33 a	451	0.00	0.04 a
10^{-5} M	-	14	47 a	35 a	517	0.22	0.04 a
0	-	21	70 b	46 b	330	0.23	1.41 b
-	10^{-2} M	10 a	48 a	36	584 c	0.07	0.54
-	10^{-3} M	16 ab	54 ab	36	451 b	0.30	0.54
-	0	22 b	62 b	41	264 a	0.08	0.42

[1]Values are the mean of two replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 8.--Atrazine-phosphate combination effects on respiration and photosynthesis in sorghum.[1]

Atrazine concentration	Phosphate concentration	Leaf surface area cm^2	Plant weight mg	Plant height mm	Respiration $\mu g\ CO_2/min/g$	Net photosynthesis $\mu g\ CO_2/min/cm^2$	Total photosynthesis $\mu g\ CO_2/min/cm^2$
10^{-4} M	10^{-2} M	2 a[2]	9 a	56 a	896 b	0.00 a	0.12 a
10^{-4} M	10^{-3} M	5 ab	10 a	92 ab	454 a	0.00 a	0.06 a
10^{-4} M	0	6 ab	14 ab	116 b	551 a	0.00 a	0.68 a
10^{-5} M	10^{-2} M	6 ab	13 ab	101 b	457 a	0.02 a	0.94 a
10^{-5} M	10^{-3} M	8 b	14 ab	119 b	290 a	0.35 ab	0.86 a
10^{-5} M	0	8 b	14 ab	119 b	359 a	0.30 ab	0.80 a
0	10^{-2} M	6 b	19 b	116 b	401 a	0.82 abc	1.97 b
0	10^{-3} M	9 b	19 b	119 b	463 a	1.12 bc	2.21 b
0	0	9 b	19 b	136 b	345 a	1.52 c	2.26 b
Average effect of levels							
10^{-4} M	-	5 b	11 b	88 b	632 b	0.00 a	0.29 a
10^{-5} M	-	7 a	14 b	113 a	369 a	0.22 a	0.87 b
0	-	8 a	19 a	124 a	403 a	1.15 b	2.15 c
-	10^{-2} M	5 b	14	91 b	583	0.28	1.01
-	10^{-3} M	7 a	14	110 ab	403	0.49	1.04
-	0	8 a	16	124 a	418	0.61	1.25

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 9.--Atrazine-phosphate combination effect on respiration and photosynthesis in soybeans.[1]

Atrazine concentration	Phosphate concentration	Leaf surface area cm^2	Plant weight mg	Plant height mm	Respiration µg CO_2/min/g	Net photosynthesis µg CO_2/min/cm^2	Total photosynthesis µg CO_2/min/cm^2
10^{-4} M	10^{-2} M	0 a[2]	15 a	28 a	1764 b	0.00	0.00
10^{-4} M	10^{-3} M	3 ab	20 a	48 ab	1377 ab	0.00	0.54
10^{-4} M	0	2 ab	15 a	42 ab	1706 b	0.00	0.00
10^{-5} M	10^{-2} M	3 ab	24 ab	55 ab	1298 ab	0.10	1.78
10^{-5} M	10^{-3} M	4 ab	26 abc	79 bc	461 ab	0.00	0.12
10^{-5} M	0	6 bc	37 bc	96 c	688 ab	0.00	0.85
0	10^{-2} M	8 bc	40 c	65 bc	382 a	0.00	0.08
0	10^{-3} M	10 c	39 bc	76 bc	493 ab	0.00	1.50
0	0	7 bc	35 bc	74 bc	349 a	0.00	1.24
Average effect of levels							
10^{-4} M	-	2 a	17 a	39 a	1616 b	0.00	0.18
10^{-5} M	-	4 a	30 b	76 b	816 a	0.03	0.91
0	-	8 b	38 c	72 b	408 a	0.00	0.94
-	10^{-2} M	3	26	49	1148	0.03	0.62
-	10^{-3} M	6	28	67	777	0.00	0.72
-	0	5	30	71	914	0.00	0.70

[1]Values are the mean of two replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

Atrazine-Phosphate Combination Effect on Atrazine Uptake and Metabolism

The total amount of ^{14}C-atrazine uptake during the treatment period is reported in Tables 10 to 13 for corn, peas, sorghum and peas, respectively. The amount of the ^{14}C associated with the respective metabolic fractions is also presented for these crops in Tables 10 to 13.

In corn, the chloroform-soluble fraction which contained the unchanged atrazine and small amounts of dealkylated metabolites tended to increase with increasing phosphate levels (Table 10). Increasing the concentration of atrazine in the culture solution also tended to cause an increase in that fraction, suggesting an overload of the detoxication mechanism. The phosphate treatment also contributed to this effect suggesting that high levels of phosphate might interfere with atrazine metabolism.

Supporting this conclusion is the observation that the combination of high atrazine and phosphate resulted in a significant synergistic reduction in the amount of methanol-insoluble metabolites, the insoluble

residue assumed to be least toxic to the plants. There appears to be a reciprocal relationship between these compounds and the hydroxylated metabolites. However, since neither is toxic to plants it is difficult to envision how blocking the pathway between these two might be detrimental. It would be interesting to know how the concentration of hydroxylated metabolites subsequently affects the rate of atrazine metabolism to these same compounds. High concentration of hydroxylated compounds could shift the equilibrium in the direction of unaltered atrazine.

In corn, it appears that the interaction can best be explained by increased plant respiration and reduction in the rate of atrazine detoxication, specifically to a methanol-insoluble residue.

At the 10^{-4} M atrazine level, increasing the phosphate level tended to increase atrazine uptake (Table 10). Whereas at the 10^{-5} M atrazine level the opposite effect was apparent. However, none of the changes were significant and undoubtedly did not wholly account for the observed increase in phytotoxicity at the higher phosphate level.

Atrazine metabolism by peas followed a pattern similar to that of corn. The level of chloroform-soluble compounds tended to increase with increasing atrazine level, again suggesting an overloading effect on the detoxication system (Table 11). The lowest value was again for the low atrazine treatment alone. The reciprocal relationship between hydroxylated compounds and methanol-insoluble metabolites was also apparent as in corn.

The least conversion to methanol-insoluble metabolites occurred in the combination treatments with 10^{-2} M phosphate. An increase in the available level of phosphate significantly promoted atrazine uptake at 10^{-4} M atrazine whereas increasing the phosphate level reduced the level of atrazine uptake at the 10^{-5} M atrazine concentration. Here the plants most affected had the lowest concentration of atrazine in the tissues.

The conclusions with respect to peas are similar to those for corn. The interaction seems to be the result of a promotion of respiration possibly enhanced by a disruption of atrazine metabolism. There was no significant difference between treatments in the composition of the chloroform-soluble fraction as shown in Table 14, although the hydroxylated metabolites increased with

increasing levels of phosphates. This trend was similar to that observed for the hydroxylated metabolites in the initial separation (Table 11).

There was very little difference between the treatment means in sorghum for the distribution of ^{14}C-labeled compounds in the three metabolite fractions (Table 12). Chloroform-soluble metabolites tended to be slightly higher in plants grown in 10^{-4} M atrazine solution than in plants grown in 10^{-5} M atrazine solution but the level of phosphate did not either increase or decrease the total amount of compounds in this fraction. Hydroxylated metabolites make up a slightly lower amount of the total compounds present in plants grown on 10^{-4} M compared with 10^{-5} M atrazine. Phosphate level again had no effect. The amount of methanol-insoluble metabolites was not influenced by either atrazine or phosphate level.

An analysis of the makeup of the chloroform-soluble fraction shows that there was no significant difference between treatment means for atrazine or the hydroxylated metabolites running at the origin (Table 15). There were significant differences between treatment means for the dealkylated metabolites. The combination treatments of atrazine at 10^{-4} M and phosphate at 10^{-2} M

and 10^{-3} M contained larger amounts of these dealkylated metabolites than either treatment with atrazine alone. The small magnitude of the difference makes this statistically significant result of doubtful importance as a causal factor to the observed decrease in plant growth.

Atrazine uptake by sorghum was not linear with concentration in the culture medium (Table 12). Whereas there was a tenfold difference in atrazine concentration between the first three and second three treatments, there is only a three-fold difference in atrazine uptake. At any given level of atrazine, phosphate had no significant effect on atrazine uptake, although it tended to increase uptake at the 10^{-4} M atrazine level. The failure of uptake to be linear suggests that it may be an active process in sorghum.

Atrazine metabolism data for soybean clearly reflects the limited ability of this species to convert atrazine to other compounds (Table 13). Although there are no significant differences between the treatment means for chloroform-soluble compounds, a higher percentage of chloroform-soluble compounds remained in soybean plants than in the other species tested. There were no significant differences between the means for the

percentage of methanol-insoluble metabolites. The relative amount of hydroxylated metabolites is highest in the plants receiving both high atrazine and phosphate but the lowest relative amount is found in plants receiving 10^{-5} M atrazine in combination with 10^{-3} M phosphate. Only these two extremes are significantly different from each other. There were no significant differences between treatment means for the component metabolites of the chloroform-soluble fraction (Table 16). As with sorghum, the metabolism study did not produce any information to explain the atrazine phosphate interaction on plant growth.

Atrazine uptake by soybean was linear with concentration. Phosphate at 10^{-2} M inhibited the uptake of atrazine from a solution containing 10^{-5} M and 10^{-4} M atrazine compared with phosphate at lower levels (Table 13). The combination treatment of 10^{-2} M phosphate and 10^{-4} M atrazine was reduced in terms of plant growth compared to the other two treatments containing 10^{-4} M atrazine despite the lower atrazine uptake of these plants in the combination treatment. Thus a phosphate affect on atrazine uptake did not explain the atrazine-phosphate interaction on growth.

Table 17 shows the effect of the assay procedure on ^{14}C-atrazine recovery during the extraction procedure. Because a fairly constant amount of ^{14}C-atrazine was associated with each milligram of dry material extracted, the weight of each sample influenced ^{14}C-atrazine recovery. The label found in the insoluble fraction probably represents absorption rather than conversion during the extraction procedure. Since label in methanol-insoluble metabolites generally increases on a unit weight basis as sample weight increases, the data in Tables 10 through 13 represent a real conversion by the plant to insoluble residue.

Recovery of total label appears to be satisfactory.

Multiple thin-layer chromatography of the metabolites remaining at the origin showed most of the radioactivity to be present in hydroxyatrazine. Two other distinct metabolites of unknown identity were also present.

TABLE 10.--Atrazine-phosphate combination effects on atrazine uptake and metabolism in corn.[1]

Atrazine concentration	Phosphate concentration	Atrazine uptake µg/g dry weight	Insoluble residue percent of total	Hydroxylated metabolites percent of total	Chloroform-soluble metabolites percent of total
10^{-4} M	10^{-3} M	841 b[2]	9 a	75 b	16
10^{-4} M	10^{-3} M	614 b	23 b	65 a	11
10^{-4} M	0	574 b	17 b	72 ab	11
10^{-5} M	10^{-2} M	60 a	22 b	65 a	13
10^{-5} M	10^{-3} M	80 a	21 b	69 ab	11
10^{-5} M	0	90 a	19 b	72 ab	9
Average effect of levels					
10^{-4} M	-	677 a	17 a	71	13
10^{-5} M	-	77 b	21 b	69	11
0					
-	10^{-2} M	451	15 a	70	14
-	10^{-3} M	347	22 b	67	14
-	0	332	18 ab	72	10

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 11.--Atrazine-phosphate combination effects on atrazine uptake and metabolism in pea.[1]

Atrazine concentration	Phosphate concentration	Atrazine uptake µg/g dry weight	Insoluble residue percent of total	Hydroxylated metabolites percent of total	Chloroform-soluble metabolites percent of total
10^{-4} M	10^{-3} M	301 d[2]	29	61	11
10^{-4} M	10^{-3} M	199 cd	54	40	7
10^{-4} M	0	172 bc	49	44	8
10^{-5} M	10^{-2} M	41 a	33	59	8
10^{-5} M	10^{-3} M	60 ab	48	41	11
10^{-5} M	0	79 abc	41	55	4
Average effect of levels					
10^{-4} M	-	224 a	44	48	8
10^{-5} M	-	60 b	41	52	8
0					
-	10^{-2} M	171	31	60	9
-	10^{-3} M	129	51	41	9
-	0	126	45	50	6

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 12.--Atrazine-phosphate combination effects on atrazine uptake and metabolism in sorghum.[1]

Atrazine concentration	Phosphate concentration	Atrazine uptake µg/g dry weight	Insoluble residue percent of total	Hydroxylated metabolites percent of total	Chloroform-soluble metabolites percent of total
10^{-4} M	10^{-3} M	839 b[2]	35	59	6
10^{-4} M	10^{-3} M	858 b	34	59	7
10^{-4} M	0	596 ab	36	57	7
10^{-5} M	10^{-2} M	202 a	35	60	5
10^{-5} M	10^{-3} M	298 a	35	60	6
10^{-5} M	0	290 a	29	65	6
Average effect of levels					
10^{-4} M	-	764 a	35	58	7
10^{-5} M	-	264 b	33	62	6
0					
-	10^{-2} M	521	35	60	6
-	10^{-3} M	578	34	59	7
-	0	443	33	61	6

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 13.--Atrazine-phosphate combination effects on atrazine uptake and metabolism in soybean.[1]

Atrazine concentration	Phosphate concentration	Atrazine uptake µg/g dry weight	Insoluble residue percent of total	Hydroxylated metabolites percent of total	Chloroform-soluble metabolites percent of total
10^{-4} M	10^{-3} M	372 b[2]	28	52 b	21
10^{-4} M	10^{-3} M	489 c	51	32 ab	17
10^{-4} M	0	409 bc	47	34 ab	19
10^{-5} M	10^{-2} M	36 a	35	46 ab	18
10^{-5} M	10^{-3} M	54 a	44	28 a	28
10^{-5} M	0	54 a	37	47 ab	17
Average effect of levels					
10^{-4} M	-	423 a	42	39	19
10^{-5} M	-	48 b	39	40	21
0					
-	10^{-2} M	204	32	49 a	19
-	10^{-3} M	271	48	30 b	23
-	0	231	42	40 ab	20

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 14.--Atrazine-phosphate combination effects on the composition of the chloroform-soluble metabolite fraction of pea.[1]

Atrazine concentration	Phosphate concentration	Per cent of total ^{14}C-atrazine taken up		
		Atrazine	Hydroxylated metabolites	Dealkylated metabolites
10^{-4} M	10^{-2} M	3.1	5.8	1.6
10^{-4} M	10^{-3} M	1.8	3.3	0.8
10^{-4} M	0	2.3	3.9	0.8
10^{-5} M	10^{-2} M	0.6	3.1	0.5
10^{-5} M	10^{-3} M	2.6	4.4	2.3
10^{-5} M	0	0.9	2.0	1.0
Average effect of levels				
10^{-4} M	-	2.4	4.3	1.1
10^{-5} M	-	1.4	3.1	1.3
0				
-	10^{-2} M	1.9	4.4	1.1
-	10^{-3} M	2.2	3.8	1.6
-	0	1.6	2.9	0.9

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

TABLE 15.--Atrazine-phosphate combination effects on the composition of the chloroform-soluble metabolite fraction of sorghum.[1]

Atrazine concentration	Phosphate concentration	Per cent of total ^{14}C-atrazine taken up		
		Atrazine	Hydroxylated metabolites	Dealkylated metabolites
10^{-4} M	10^{-2} M	0.8	4.9	0.3 b[2]
10^{-4} M	10^{-3} M	0.8	5.8	0.3 b
10^{-4} M	0	0.3	5.8	0.2 ab
10^{-5} M	10^{-2} M	0.3	4.4	0.2 ab
10^{-5} M	10^{-3} M	0.3	4.8	0.1 a
10^{-5} M	0	0.4	5.0	0.2 ab
Average effect of levels				
10^{-4} M	-	0.6	5.5	0.2 a
10^{-5} M	-	0.4	4.7	0.3 b
0				
-	10^{-2} M	0.6	4.6	0.2
-	10^{-3} M	0.5	5.3	0.2
-	0	0.4	5.4	0.2

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

[2]Means with common letters are not significantly different at the 5% level by the Duncan Multiple Range Test.

TABLE 16.--Atrazine-phosphate combination effects on the composition of the chloroform-soluble metabolite fraction of soybean.[1]

Atrazine concentration	Phosphate concentration	Per cent of total ^{14}C-atrazine taken up		
		Atrazine	Hydroxylated metabolites	Dealkylated metabolites
10^{-4} M	10^{-2} M	9.1	10.3	1.9
10^{-4} M	10^{-3} M	6.7	9.8	3.1
10^{-4} M	0	7.4	10.3	3.7
10^{-5} M	10^{-2} M	5.2	8.4	5.9
10^{-5} M	10^{-3} M	7.0	8.1	6.0
10^{-5} M	0	5.4	9.5	4.5
Average effect of levels				
10^{-4} M	-	7.7	10.1	2.9
10^{-5} M	-	5.9	8.6	5.5
0				
-	10^{-2} M	7.2	9.3	3.9
-	10^{-3} M	6.9	8.9	4.6
-	0	6.4	9.9	4.1

[1]Values are the mean of 2 replications which contained seedlings germinated from 10 seeds.

TABLE 17.--The effect of assay procedure on ^{14}C-atrazine recovery during extraction procedure.[1]

Species	Percent of label in fraction: Chloroform soluble	Chloroform insoluble	Insoluble residue[2]	Percent of total ^{14}C recovered
Corn	45	34	21	77
Sorghum	68	29	3	99
Soybean	60	33	7	86
Pea	65	22	13	81

[1]Values are the mean of two replicate extractions.

[2]Variability is due to the amount of material extracted. A constant amount of label, 0.045 disintegrations per minute per milligram dry weight of extracted material was absorbed.

Atrazine Effect on ^{32}P Uptake and Distribution in Corn

Autoradiographs from the phosphate uptake study are shown in Figures 5 and 6. Figure 5 shows plants which received 10^{-2} M phosphate with and without atrazine. Phosphate is seen to be distributed uniformly throughout the plants in both treatments with no apparent difference between treatments. Shown in Figure 6 are plants which did not receive phosphate. These plants receiving or not receiving atrazine also show a uniform phosphate distribution with little apparent difference between treatments.

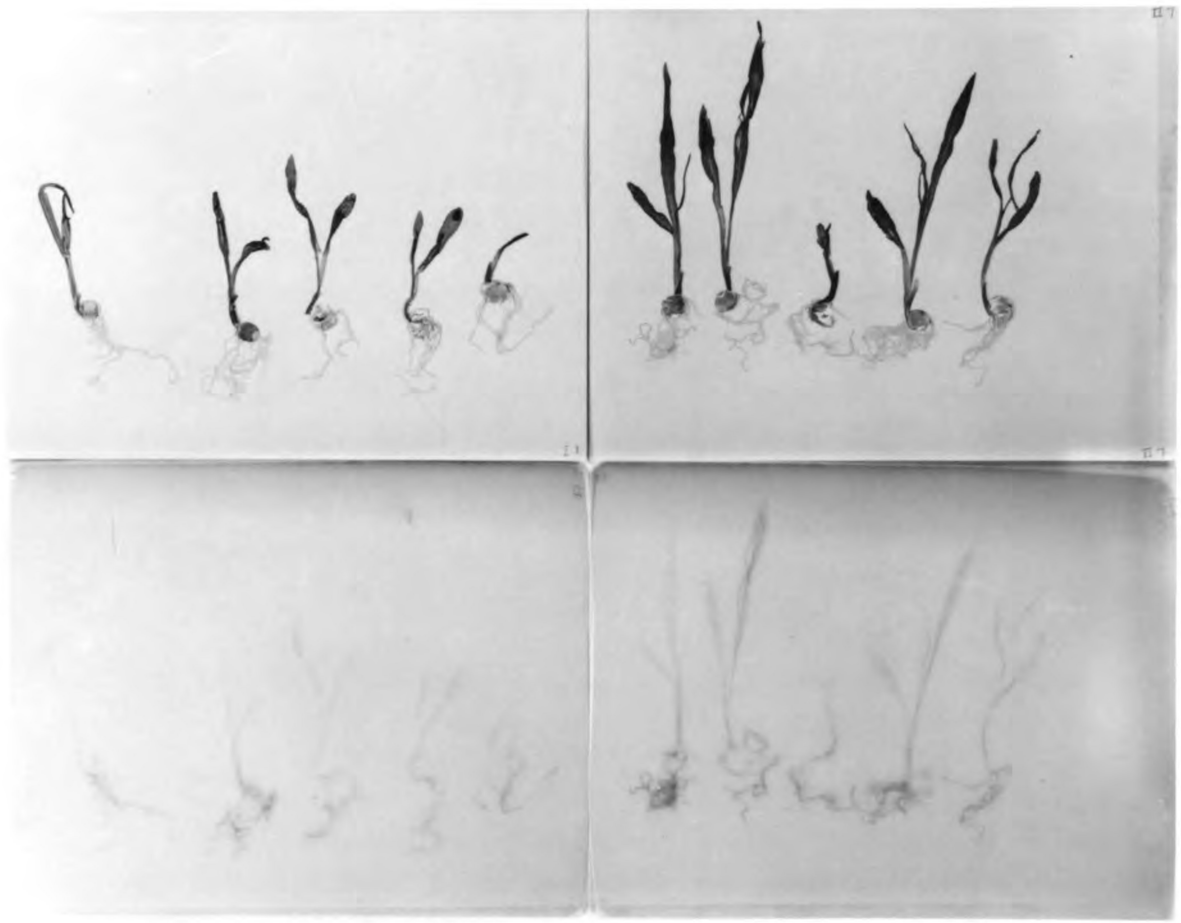

Fig. 5.--The effect of atrazine on ^{32}P uptake of corn plants grown in 10^{-2} M phosphate. Autoradiograph on left shows plants which received atrazine treatment at 10^{-4} M. Autoradiograph on right shows plants which received no atrazine.

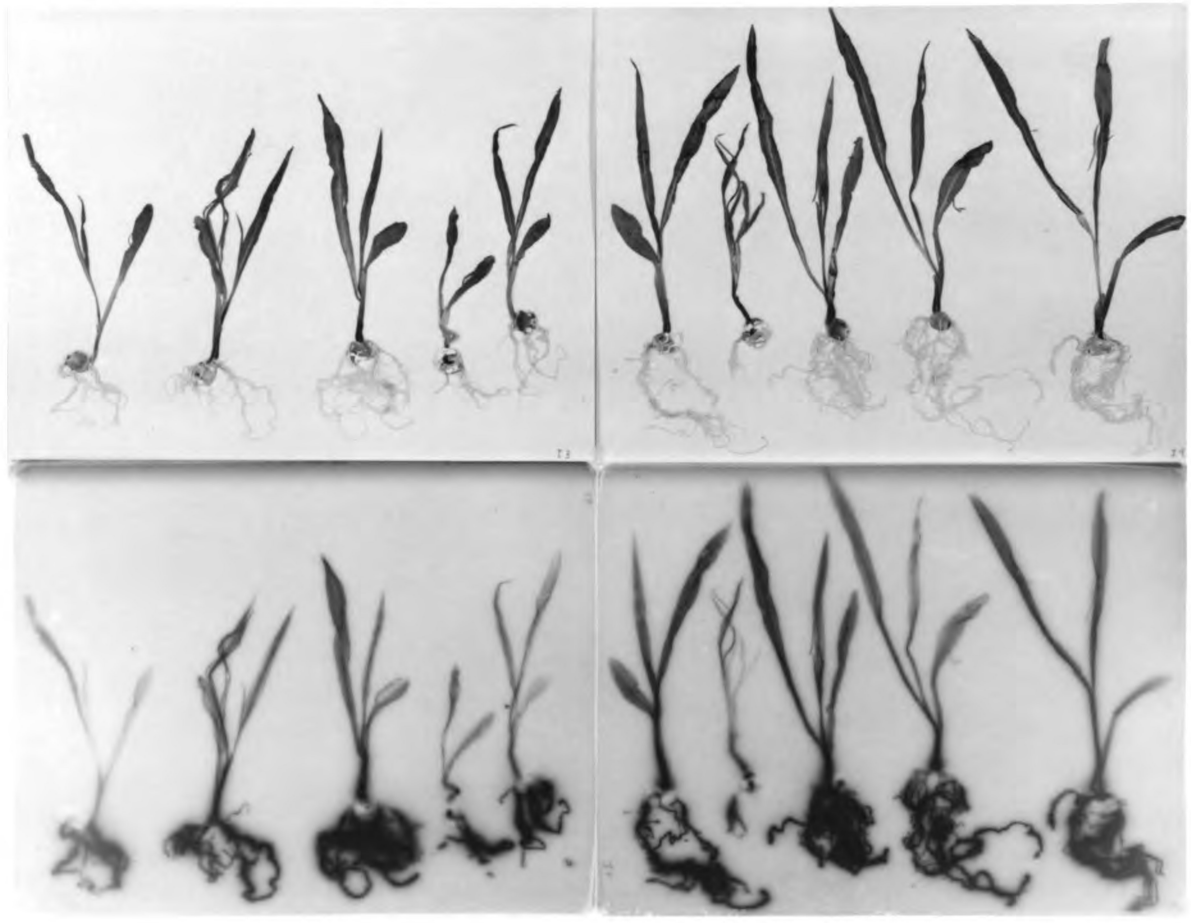

Fig. 6.--The effect of atrazine on ^{32}P uptake of corn plants grown without phosphate. Autoradiograph on left shows plants which received atrazine treatments at 10^{-4} M. Autoradiograph on the right shows plants which received no atrazine.

It was not possible to compare phosphate uptake with and without phosphate in solution. Phosphate uptake was no longer linear with concentration at 10^{-2} M.

Table 18 shows the data for actual amounts of ^{32}P present in the plant material. As the t-test indicates there are not significant differences at the 5% level between plants treated and not treated with atrazine. The differences are approaching significance in the phosphate-treated plants but it does not appear that effects on phosphate uptake can explain the atrazine-phosphate interaction.

TABLE 18.--The effect of atrazine on ^{32}P uptake.[1]

Atrazine concentration	Phosphate concentration	^{32}P counts/minute
10^{-4} M	10^{-2} M	432
0	10^{-2} M	652
10^{-4} M	0	7458
0	0	6923

[1]Values are the mean of 4 samples each containing 2 or 3 corn shoots.

SUMMARY AND CONCLUSIONS

Of the five crop species examined, corn, peas, sorghum, and soybeans showed greater reduction in plant growth following atrazine treatment if high levels of phosphate were also present in the treatment solution. Only in the case of peas was the high level, 10^{-2} M phosphate, in the treatment solution significantly phytotoxic by itself. Barley did not show the atrazine-phosphate combinations effect evident in corn, peas, sorghum, and soybeans.

The enhanced phytotoxicity of atrazine in the presence of phosphate appeared to be related to a synergistic increase in respiration of corn, peas, sorghum, and soybean plants receiving the combination treatment. The atrazine induced inhibition of photosynthesis in these species was not synergistically enhanced by the additional presence of phosphate.

Although the trends were not statistically significant at the 5% level, at the 10^{-5} M atrazine level, increasing levels of phosphate tended to reduce total

atrazine uptake during the treatment period. For corn, peas, and soybean this trend was reversed at the 10^{-4} M atrazine level.

The percent of chloroform-soluble metabolites, including the parent atrazine, increased with increasing atrazine and phosphate levels in corn and peas. If this effect were of sufficient magnitude it could explain enhanced phytotoxicity. The presence of phosphate with the atrazine treatment reduced the metabolism of atrazine to a methanol-insoluble residue and increased the accumulation of hydroxylated metabolites in corn, peas, and soybeans. It is difficult to envision how this effect could explain the altered phytotoxicity unless the assumption on the phytotoxicity of these metabolites are erroneous or this block in metabolism also affected the rate of conversion of atrazine to hydroxylated metabolites. There did not appear to be any affect of phosphate on atrazine metabolism by sorghum.

Atrazine did not affect the uptake or distribution of ^{32}P by corn.

The enhanced phytotoxicity of atrazine in the presence of high levels of phosphate can best be explained by an interaction on the rate of respiration. The

increased respiration could be due to an interaction effect on the respiration apparatus or by an increase in the internal concentration of atrazine resulting from a slight increase in uptake and a slight decrease in metabolism of atrazine in the presence of high levels of phosphate. These alternatives are not mutually exclusive.

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