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# THE EFFECT OF HERBICIDES, PLANT GROWTH REGULATORS AND OTHER COMPOUNDS ON PHENYLALANINE AMMONIA-LYASE ACTIVITY

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**Key Word Index**—*Setaria viridis*; Gramineae; phenylalanine ammonia-lyase; herbicides; plant growth regulators; product analogs.

**Abstract**—The *in vitro* and *in vivo* effects of a number of herbicides and plant growth regulators on phenylalanine ammonia-lyase (PAL) activity were investigated. The most effective *in vitro* inhibitors were product analogs, *t*-cinnamic and *p*-coumaric acids, and carbonyl reagents, hydroxylamine and nitromethane. Application of the herbicides diuron, dalapon, amiben, and chloropropham, to plants resulted in a decrease in the intracellular concn of PAL. The inhibitory effect of alachlor was found to be dose-responsive and somewhat specific. A correlation between PAL inhibition and herbicidal activity was observed for hydroxylamine. The cytokinin, pyranyl benzyladenine, (PBA) increased PAL activity in pigweed. The possibility of developing herbicides acting through PAL inhibition is discussed.

## INTRODUCTION

THE POSSIBILITY of developing a herbicide acting through selective inhibition of phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) was discussed in a previous paper.[1] This paper describes the effect of several herbicides and plant growth regulators on the *in vivo* and *in vitro* activity of PAL. The relationship between PAL inhibition and herbicidal activity is explored.

## RESULTS

The effects of various herbicides, plant growth regulators, and carbonyl compounds† on the activity of PAL in plants are shown in Table 1. The compounds were applied 17·5 hr before harvesting as described in the Experimental Section using a solution of 3 mg active material/ml in 10% acetone. The plants used were green foxtail (GF, *Setaria viridis* Beauv.), pigweed (PW, *Amaranthus retroflexus* L.), yellow nutsedge (YNS, *Cyperus esculentus* L.), and purple nutsedge (PNS, *C. rotundus* L.). Some compounds produced the same effect in all the species tested, while others produced opposite effects in different species. The extraction procedure and assay system were carefully checked and found to be quite reproducible. The effects observed, however, were somewhat variable due to slight differences in plant age and difficulties in reproducibly applying the compounds. Among the most

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† Compound listed: 2,4-D, 2,4-dichlorophenoxyacetic acid; Diuron, 3-[3,4-dichlorophenyl]-1,1-dimethylurea; Dalapon, 2,2-dichloropropionic acid; Chloropropham, isopropyl *N*-[3-chlorophenyl] carbamate; Chloramben, 3-amino-2,5-dichlorobenzoic acid; Chlorflurecol, methyl-2-chloro-9-hydroxyflurene-9-carboxylate; Alachor, 2-chloro-2′,6′-diethyl-*N*-[methoxymethyl]-acetanilide; Endothall, 7-oxabicyclo-[2,2,1]-heptane-2,3-dicarboxylic acid; Diallate, S-2,2,3-trichloroallyl-diisopropylthiocarbamate; Fenuron, 3-phenyl-1,1-dimethyl urea.

[1] JANGAARD, N. O. (1974) *Phytochemistry* **13**, 1765.

effective inhibitors of PAL were 2,4-D, diuron, dalapon, and chloropropham. Endothall, alachlor, and diallate were effective inducers of PAL in pigweed.

TABLE 1. EFFECTS OF VARIOUS PLANT-ACTIVE COMPOUNDS ON *in vivo* PAL ACTIVITY LEVELS

| Compound | Species* | Effect | Compound | Species | Effect |
|---|---|---|---|---|---|
| Abscisic acid | GF | +103% (9) | Alachlor | GF | −61% (4) |
| | YNS | −74% (2) | | PW | +65% (8) |
| | PNS | −42% (2) | | | |
| | | | Endothall | GF | 0 (5) |
| 2,4-D | GF | −45% (3) | | PW | +267% (6) |
| | PW | −49% (3) | | | |
| Diuron | GF | −40% (2) | Hydroxylamine | GF | −41% (7) |
| | PW | −50% (2) | Nitromethane | GF | 0 |
| Dalapon | GF | −30% (2) | Semicarbazide | GF | 0 |
| | PW | −49% (3) | | | |
| | | | Sodium Borohydride | GF | 0 |
| Chloropropham | GF | −39% (8) | | | |
| | PW | −52% (10) | Phenylhydrazine | GF | −34% (3) |
| | YNS | −58% (2) | | | |
| | PNS | −55% (2) | Sodium Cyanide | GF | −48% (3) |
| Chloramben | GF | −20% (3) | Diallate | GF | −54% (3) |
| | PW | −33% (3) | | PW | +120% (3) |
| Chlorflurecol | GF | −40% (4) | Fenuron | GF | −60% (4) |
| | PW | −41% (5) | | PW | 0 |
| | PNS | −45% (1) | | | |

* GF = *Setaria viridis* (L.) Beauv., green foxtail. PW = *Amaranthus retroflexus* L., pigweed (redroot). YNS = *Cyperus esculentus* L., yellow nutsedge. PNS = *Cyperus rotundus* L., purple nutsedge.

The effect of varying concentrations of alachlor on PAL activity in green foxtail was then investigated. Solutions containing 0·5, 1, 2, and 3 mg of the compound per ml were applied to 4-week-old plants and the PAL activity and protein concentration measured 17 h later. Two controls were used and each group was run in triplicate. The effects observed, going from lowest to highest dose, were none, 19, 40 and 73% decrease in specific activity. The effect of the inhibitor on the *in vivo* activity of three other enzymes in addition to PAL is shown in Table 2. The IDH and G-6-PDH activities were slightly increased by alachor while PAL and TAL activities were decreased by 61 and 58%, respectively.

TABLE 2. ALACHLOR EFFECT ON *in vivo* ACTIVITY OF FOUR GREEN FOXTAIL ENZYMES

| Group* | Protein mg/ml | Isocitric DeH'ase Sp. Act. | % Diff. | G-6-P DeH'ase Sp. Act. | % Diff. | PAL Sp. Act. | % Diff. | TAL Sp. Act. | % Diff. |
|---|---|---|---|---|---|---|---|---|---|
| Control | 2·01 | 237·0 | | 228·7 | | 31·5 | | 5·4 | |
| Alachlor† | 2·12 | 260·4 | +10% | 239·4 | +5% | 12·3 | −61% | 2·3 | −58% |

* Average of two experiments.
† Treated with 3 mg/ml and harvested 16 hr later.

Figure 1 illustrates the time course of the effect of pyranyl benzyladenine (PBA) on PAL in pigweed. Four hours following treatment, the specific activity of PAL was the same in both groups. Following this, PAL levels increased greatly in the treated plants with the maximum effect observed 48 hr after treatment.

The effect of a number of compounds on the *in vitro* activity of a corn acetone PAL preparation is illustrated in Table 3. The value shown is the mean response and the

number in parentheses indicates the number of times the compound was tested. A value of less than 15% was considered to be no effect. The most effective inhibitors were *t*-cinnamic acid and *p*-coumaric acid. Compounds such as 2,4-D and chloropropham which decreased the *in vivo* activity of PAL, had no effect on the *in vitro* activity of the enzyme.

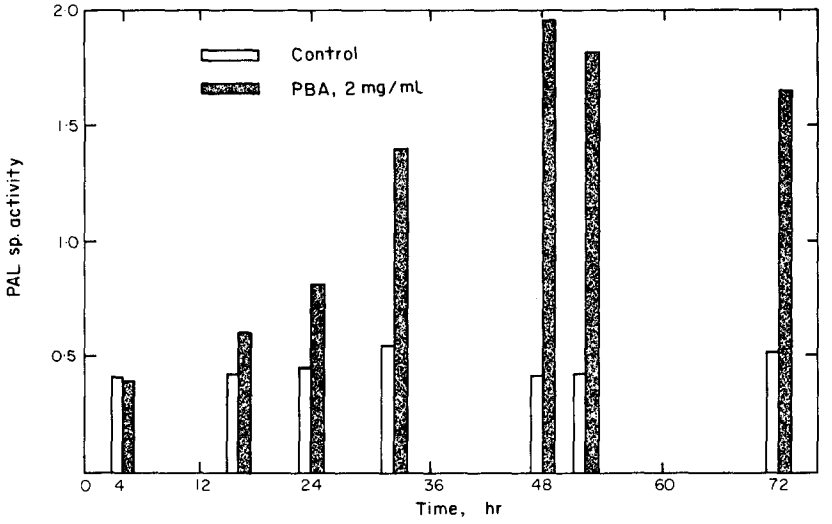


FIG. 1. EFFECT OF PBA APPLICATION ON *in vivo* ACTIVITY OF PAL IN PIGWEED.
The plants were homogenized (1 g fresh wt/10 ml borate buffer) as described in the Experimental section. The homogenate was centrifuged for 20 min at 28 700 *g* and the supernatant solution was used for the analyses.

The effect of the carbonyl reagents phenylhydrazine, sodium cyanide, and sodium borohydride on the *in vitro* activity of PAL from green foxtail is shown in Fig. 2. Sodium cyanide was the most effective inhibitor of the three, giving a 96% inhibition at a 1·0 mM concn. Figure 3 illustrates the effect of varying concentrations of hydroxyl-amine and nitromethane on green foxtail PAL activity. Both compounds are effective inhibitors, giving approximately 90% inhibition at a concentration of 0·5 mM.

TABLE 3. *In vitro* INHIBITION OF CORN ACETONE PAL PREPARATION

| Compound | Conc (μM) | Effect | Compound | Conc (μM) | Effect |
|---|---|---|---|---|---|
| *p*-Coumaric | 10 | −38% (2) | Gibberellic Acid | 10 | −18% (2) |
| acid | 100 | −71% (5) | | 100 | −27% (2) |
| *t*-Cinnamic | 10 | −39% (2) | PBA | 10 | −23% (2) |
| acid | 100 | −83% (2) | | 100 | −27% (2) |
| Caffeic acid | 10 | −24% (2) | Abscisic Acid | 10 | 0 |
| | | | | 100 | −28% (2) |
| Ferulic acid | 10 | 0 | Alachlor | 10 | 0 |
| | | | | 100 | 0 |
| Salicylic acid | 10 | 0 | | | |
| Syringic acid | 10 | −30% (2) | 2,4-D | 100 | 0 |
| Sinapic acid | 10 | −29% (2) | Chloropropham | 100 | 0 |
| Vanillic acid | 10 | 0 | Endothall | 10 | 0 |
| Tri-Iodo- | 10 | −24% (2) | Chlorflurecol | 10 | 0 |
| Benzoic acid | 100 | −36% (5) | | 100 | −34% (3) |

The L-phenylalanine concentration in the reaction mixture was $5 \times 10^{-4}$ M, the incubation temp. was 38°, and the incubation time was 2·5 hr. Each assay was run in triplicate.

The effect of applying hydroxylamine to young green foxtail plants is shown in Table 4. The plants were sprayed until drenched with a surfactant solution (controls) or the same solution containing 1, 2, or 4 mg hydroxyl-amine/ml. Twenty hr later the appearance of the plants was noted and a sample taken for the determination of PAL activity and protein concentration. The same procedure was reported 72 hr following treatment. At 20 hr there was a positive correlation between the degree of plant damage observed, the amount of hydroxylamine applied, and the degree of PAL inhibition. Between 20 and 72 hr the plants receiving the highest dosage died. At this time, the degree of PAL inhibition and the degree of plant damage had increased markedly in the plants sprayed with the solution containing 2 mg hydroxylamine/ml.

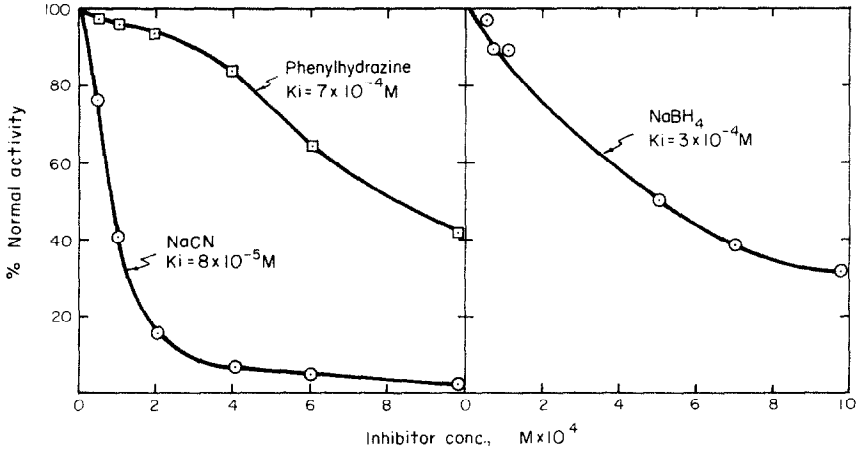


FIG. 2. INHIBITION OF GREEN FOXTAIL PAL BY SODIUM CYANIDE, SODIUM BOROHYDRIDE AND PHENYLHYDRAZINE.
Experimental details are in the text.

Table 5 illustrates the relative sensitivity of PAL and TAL from green foxtail to *in vitro* inhibition by hydroxylamine and nitromethane. The inhibitors were added to the reaction mixture to give a final concentration of $5 \times 10^{-5}$ M and $2 \times 10^{-4}$ M. The two enzymes were equally sensitive to inhibition by these compounds.

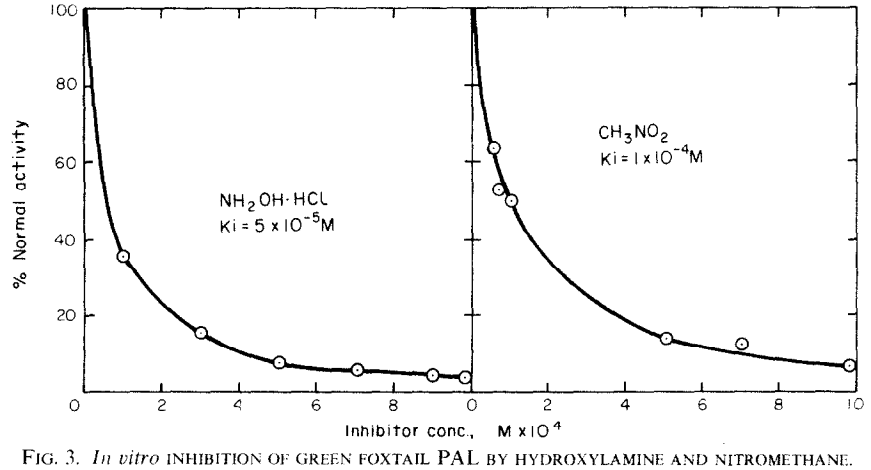


FIG. 3. *In vitro* INHIBITION OF GREEN FOXTAIL PAL BY HYDROXYLAMINE AND NITROMETHANE.

TABLE 4. EFFECT OF HYDROXYLAMINE ON GROWTH OF GREEN FOXTAIL AND ON *in vivo* PAL LEVELS

| Hours following treatment | $NH_2OH$ added (mg/ml) | Cinnamic acid produced (μmol/ml) | Protein (mg/ml) | Sp. act. | Diff. | Appearance of plants |
|---|---|---|---|---|---|---|
| 20 | 0 | 26·2 | 0·93 | 28·2 | — | Normal |
| | 1 | 20·2 | 0·99 | 20·3 | −28% | Slight dehydration |
| | 2 | 19·5 | 1·06 | 18·4 | −35% | Chlorotic |
| | 4 | 21·3 | 1·90 | 11·2 | −60% | Withered |
| 72 | 0 | 21·0 | 109 | 19·3 | — | Normal |
| | 1 | — | — | — | — | None available |
| | 2 | 8·5 | 1·38 | 6·2 | −68% | Severely withered |
| | 4 | — | — | — | — | Plants dead |

## DISCUSSION

The compounds producing the most *in vitro* PAL inhibition were either products, *t*-cinnamic acid and *p*-coumaric acid, or carbonyl reagents, hydroxyl-amine and nitromethane. The product inhibitors behaved as classical competitive inhibitors and the corn green foxtail and pigweed PAL preparations were similar in this respect to PAL from other sources.[2–7] Both *t*-cinnamic acid and *p*-coumaric acid also inhibited TAL from green foxtail. The carbonyl reagents were probably active as a result of their ability to covalently react with the dehydroalanyl residue reported to be present in the active site of the enzyme.[7–10] The kinetic properties of the enzyme in the presence of these inhibitors is consistent with this view. The inhibition produced by these compounds was non-competitive, irreversible, and could be increased by preincubating the enzyme with the inhibitor. No evidence was obtained to suggest that PAL from green foxtail is an allosteric enzyme.[5]

TABLE 5. *In vitro* INHIBITION OF PAL AND TAL FROM GREEN FOXTAIL BY HYDROXYLAMINE AND NITROMETHANE

| Inhibitor | Inhibitor concn (μM) group | PAL activity ΔA° 270 nm | PAL activity % Inhibition | TAL activity ΔA° 330 nm | TAL activity % Inhibition |
|---|---|---|---|---|---|
| $NH_2OH$ | 0 | 1·042 | — | 0·168 | — |
| | 50 | 0·828 | −21% | 0·141 | −16% |
| | 200 | 0·356 | −65% | 0·092 | −55% |
| $MeNO_2$ | 0 | 0·427 | — | 0·160 | — |
| | 50 | 0·213 | −50% | 0·064 | −60% |
| | 200 | 0·091 | −79% | 0·008 | −95% |

The *in vivo* activity of PAL is known to be affected by a number of variables.[11–13] Compounds that intercalate with deoxyribonucleic acid in peas produce increased levels of PAL

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activity and increase the rate of pisatin synthesis.[14,15] In this paper PBA (pyranyl benzyladenine), a cytokinin, was found to induce PAL activity in pigweed. Kinetin has a small stimulatory effect on PAL activity in grapefruit callus.[16] Cytokinins stimulate isoflavone and anthocyanogen synthesis in clover,[17] an action which may be mediated through PAL induction. Gibberellic acid promotes lignification and induces PAL in several plant species.[16–19] Ethylene also increases PAL activity.[20–22] Abscisic acid depresses lignin and phenolic biosynthesis,[23] a finding that could be explained by PAL inhibition. In this paper, abscisic acid was found to increase PAL activity in green foxtail and to decrease it in yellow and purple nutsedge. Abscisic acid has been reported to produce both an inhibition and a stimulation of PAL activity *in vivo*.[13,16,24] 2,4-D decreased PAL activity in both pigweed and green foxtail.

A number of herbicides, affected the *in vivo* activity of PAL. Diuron, dalapon, Amiben, CIPC, and Maintain (CF. 125) consistently produced a PAL inhibition in all four of the species tested. It is difficult to assess the relationship between the herbicidal activity of these compounds and their inhibitory effect on PAL. It should be noted that there was a dose-response relationship between the amount of alachlor applied to green foxtail and the inhibition of PAL obtained (Table 1). It was also found that alachlor application could significantly affect PAL and TAL activity without affecting the activity of isocitric dehydrogenase, a citric acid cycle enzyme, or glucose-6-phosphate dehydrogenase, an enzyme of glucose metabolism.

One of the reasons for selecting PAL as a possible target for herbicide design is that this enzyme has not been reported to occur in mammalian cells.[25,26] If an inhibitor highly specific for PAL can be designed, it may display a low level of mammalian toxicity. However, it should be noted that histidine ammonia-lyase, an enzyme found in mammalian cells, appears to contain a dehydroalanyl moiety in its active site,[27–29] as does PAL.[7–10] The effect of PAL inhibitors on histidine ammonia-lyase should be ascertained therefore prior to the widespread use of such an agent.

Another reason for selecting PAL inhibition as an approach to herbicide design was the hope of obtaining a selective herbicide. It was hoped to achieve this by selectively inhibiting PAL activity while leaving TAL activity unchanged. Those plants containing primarily PAL activity would be adversely affected by an inhibitor of this type, while those containing both PAL and TAL activity, including corn, wheat, sorghum, oats, rice, sugar cane and barley, would be much less sensitive.[1,25,26] However, the findings that PAL and TAL are equally inhibited by some compounds *in vivo* (Table 2) and *in vitro* (Table 5), that PAL

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and TAL from green foxtail cannot be separated easily,[1] and the literature report that the deamination of both L-phenylalanine and L-tyrosine is catalyzed at a common catalytic site on the PAL from maize,[2] suggest that it may not be possible to achieve selectivity in this way.

The question of the relationship between PAL inhibition and plant survival is difficult to answer. If one observed a 90% inhibition of PAL activity and no deleterious effect on plant growth, then it can be concluded that PAL activity is non-essential. This was never observed. In this study a positive correlation between herbicidal activity and PAL inhibition was observed (Tables 1, 2 and 4). This finding, together with the general background showing the relationship between PAL activity and lignification,[12] makes it appear reasonable to operate on the assumption that PAL inhibitors may have herbicidal or plant growth regulatory activity.

## EXPERIMENTAL

The PAL and TAL assays and the corn acetone powder preparation were described in the previous paper.[1] Isocitric dehydrogenase and glucose-6-phosphate dehydrogenase were assayed according to standard methods.[30]

In vivo *enzyme tests.* Three to five-week old plants were sprayed until drenched with a 10% acetone soln (0·5% Tween 80 and 0·5% Span 20) containing the test compound. The plants were routinely sprayed at 16.00 hr and harvested the following morning at 09:30 hr. The plants were washed, weighed, minced, and homogenized in a Virtis homogenizer. Cold 0·1 M borate buffer (pH 8·8, 5 mM glutathione) was used at a ratio of 1 g plant material per 4 ml buffer. The homogenizer was run at the 60 setting and run for a period of 45 sec. The homogenate was filtered through cheesecloth and the filtrate was centrifuged in a refrigerated centrifuge for 15 min at 29,000 *g*. The temperature was maintained at 5°. The supernatant solution was then decanted into chilled test tubes and used for protein[31] and enzyme assays.[1]

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