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Effects of Glyphosate on Metabolism of Phenolic Compounds

V. L-α-AMINOOXY-β-PHENYLPROPIONIC ACID AND GLYPHOSATE EFFECTS ON PHENYLALANINE AMMONIA-LYASE IN SOYBEAN SEEDLINGS[1]

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ABSTRACT

The phenylalanine ammonia-lyase (PAL) inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP) was root-fed to light-exposed soybean seedlings alone or with glyphosate [*N*-(phosphonomethyl)glycine] to test further the hypothesis that PAL activity is involved in the mode of action of glyphosate. Extractable PAL activity was increased by 0.01 and 0.1 millimolar AOPP. AOPP reduced total soluble hydroxyphenolic compound levels and increased phenylalanine and tyrosine levels, indicating that *in vivo* PAL activity was inhibited by AOPP. The increase in extractable PAL caused by AOPP may be a result of decreased feedback inhibition of PAL synthesis by cinnamic acid and/or its derivatives. AOPP alone had no effect on growth (fresh weight and elongation) at either concentration, but at 0.1 millimolar it slightly alleviated growth (fresh weight) inhibition caused by 0.5 millimolar glyphosate after 4 days. Reduction of the free pool of phenylalanine by glyphosate was reversed by AOPP. These results indicate that glyphosate exerts some of its effects through reduction of aromatic amino acid pools through increases in PAL activity and that not all growth effects of glyphosate are due to reductions of aromatic amino acids.

After the molecular mode of action of the nonselective herbicide glyphosate[2] was proposed to be through inhibition of aromatic amino acid synthesis (21) data from other laboratories gave inconsistent support for this hypothesis (8, 10, 14). The hypothesis was based upon partial reversal of glyphosate's growth-inhibiting effects on duckweed and *Rhizobium japonicum* by exogenous aromatic amino acids. In seedlings or tissue cultures of higher terrestrial plants, others have found that glyphosate treatment does not always lower levels of free aromatic amino acids (14) and that feeding aromatic amino acids does not always reverse growth inhibition caused by glyphosate (8, 10). Also, Roisch and Lingens (25) found no *in vitro* effect of glyphosate on the enzymes (chorismate mutase and prephenate dehydrogenase) involved in aromatic amino acid synthesis that Jaworski (21) hypothesized were inhibited by glyphosate. They did, however, find that 3-deoxy-D-arabino-heptulosonate-7-phosphate-synthetase was inhibited *in vitro* by 10 mM glyphosate.

Induction of PAL activity has been shown to reduce aromatic amino acid pool levels sufficiently in Jerusalem artichoke callus cultures to reduce growth rates which, in turn, could be regained by feeding aromatic amino acids (9, 20). We have therefore, hypothesized that glyphosate exerts all or part of its effects through induction of PAL activity (10, 11, 16, 17). High *in vivo* levels of PAL activity might inhibit growth in three ways: (*a*) depletion of free pools of phenylalanine and possibly tyrosine (TAL activity is often associated with PAL activity), thus inhibiting protein synthesis; (*b*) production of toxic levels of ammonia, provided amination reactions do not keep pace with deaminations; and/or (*c*) increased levels of growth-inhibiting phenolic compounds.

With roots of dark-grown maize seedlings (10, 16) and axes of dark- (17) and light- (11) grown soybean seedlings, we demonstrated that glyphosate increases levels of extractable PAL activity, often before growth effects are significant. Extractable PAL activity was negatively correlated with pools of phenylalanine and tyrosine (11, 16, 17). In glyphosate-treated plants we and others have found increases in either ammonia or in free amino acids that are early amination products (11, 16, 17, 23). However, we have found that glyphosate caused a decrease in both concentration (per g fresh weight) and content (per plant) of total alcohol-soluble hydroxyphenolic compounds (as assayed with a phosphomolybdic-phosphotungstic reagent) in tissues most affected by glyphosate (11, 16, 17). Bases for several equivocations that may be made regarding these findings are: (*a*) reliability of the assay used with qualitative changes in hydroxyphenolic compounds; (*b*) levels of insoluble and undetected hydroxyphenolic compounds; (*c*) turnover of hydroxyphenolic compounds. Consequently, our findings do not unequivocally support Jaworski's (21) or our (10, 11, 16, 17) hypothesis.

We have sought to clarify this situation by examination of the effects of a PAL inhibitor on glyphosate-caused effects, reasoning that, if our hypothesis is correct, a specific PAL inhibitor should reverse the effects of glyphosate. In this paper we report our results with AOPP, an inhibitor of PAL activity (3, 4, 5) that less effectively inhibits transaminases (6). Secondarily, we have sought to add to the understanding of the relationships between PAL, its substrate(s), and its products.

MATERIALS AND METHODS

Plant Material. Seeds of *Glycine max* (L.) Merr. cv. Hill were germinated in darkness for 3 days at 25 C as previously described (11, 17). Uniform seedlings were then transferred to test solutions containing 2 mM $CaSO_4$ and the appropriate test chemical. Due to the small amount of AOPP available, seedlings used in experiments with AOPP were transferred to 50-ml tubes (2.5 cm diameter × 10 cm length) containing 30 ml of test solution (about 20 seedlings per tube). No qualitative differences in growth and biochemical parameters were found between results with this cultural technique and those used previously in control or glyphosate-treated seedlings (11, 17). Seedlings were exposed to

[1] Mississippi Agriculture and Forest Experimental Station cooperating.
[2] Abbreviations: glyphosate: *N*-(phosphonomethyl)glycine; PAL: phenylalanine ammonia-lyase (EC 4.3.1.5); TAL: tyrosine ammonia-lyase; AOPP: L-α-aminooxy-β-phenylpropionic acid; AOA: aminooxyacetic acid.

continuous white light (11) at 25 C beginning at 3 days of age. Intact axes (root and hypocotyl) or separate roots and hypocotyls were used.

Test Chemicals. High purity glyphosate (free acid form, 99.9% by weight) was provided by Monsanto Agricultural Chemicals Company. High purity AOPP (hydrobromide form) was provided by Dr. J. S. Morely of ICI Pharmaceutical Division, Macclesfield, Cheshire, U.K.

PAL Extraction and Assay. PAL activity was extracted, partially purified by precipitation, and assayed as before (11, 17). The enzyme unit used is that of Havir and Hanson (15)—1 unit (U) = 1 μmol cinnamic acid formed per min at 30 C.

Hydroxyphenolic Assays. Total alcohol-soluble hydroxyphenolic compounds were assayed as before (16) by the method of Singleton and Rossi (26).

Amino Acid Analysis. Free amino acids were extracted and assayed as before (17).

Protein Assays. Soluble protein was extracted and assayed as before (17).

Statistical Analysis. All error bars in figures are one standard error. In tables, significant differences were determined by overlap of two standard errors. All values represent means of from two to four separate determinations.

RESULTS

Growth Effects. At 0.1 mM, AOPP inhibited fresh weight gain of seedlings after 72 h of treatment (Fig. 1). No significant effect of AOPP was found on glyphosate-treated seedling fresh weight gain although fresh weight means were higher in AOPP plus glyphosate than in glyphosate treatments at all sample times. At 0.1 mM, AOPP reversed an inhibitory effect of glyphosate on longitudinal growth by 96 h, when the axes of glyphosate-treated seedlings were 152.9 ± 0.4 mm long, whereas those of seedlings treated with glyphosate and AOPP were 166.9 ± 4.0 mm long. Control and AOPP-treated axes were 170.6 ± 0.3 and 178.8 ± 7.9 mm long, respectively. AOPP at 10 μM caused no significant effects on growth when supplied alone or to glyphosate-treated seedlings.

PAL Activity. PAL activity from axes was completely inhibited *in vitro* by 10 μM AOPP. AOPP at 0.1 mM increased extractable PAL activity by all measurement criteria (Fig. 2). On a per g fresh weight or per axis basis (Fig. 2, B and C, respectively) the enhancing effect of glyphosate on extractable PAL activity was greater than that of AOPP or AOPP plus glyphosate. The effects of the three treatments on specific acitivity were similar (Fig. 2A). AOPP at 10 μM raised levels of extractable PAL activity by all measurement criteria from 24 to 72 h of treatment, however, the effects were not as pronounced as at 0.1 mM.

Soluble Hydroxyphenolics. All treatments reduced the amount of alcohol-soluble hydroxyphenolic compounds per axis from 48 to 96 h (Fig. 3).

Amino Acids. Phenylalanine content markedly increased in axes of seedlings treated with 0.1 mM AOPP (Fig. 4A). AOPP reversed the reduction of free phenylalanine levels by glyphosate to produce levels higher than control levels. Similar effects were seen on tyrosine levels. In this case, AOPP did not reverse the effect of glyphosate until after 72 h, and the enhancement by AOPP alone was lost after 72 h (Fig. 4B). Glyphosate initially increased total amino acids, but no significant effects were measurable after 24 h

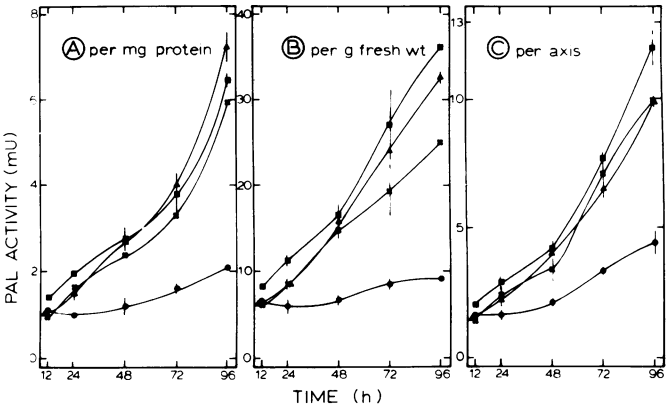

FIG. 2. PAL activities extracted from soybean axes of seedlings exposed to continuous white light and root-fed various chemicals after 3 days of dark growth. ●: Control; ■: 0.5 mM glyphosate; ▲: 0.1 mM AOPP; ×: 0.5 mM glyphosate and 0.1 mM AOPP.

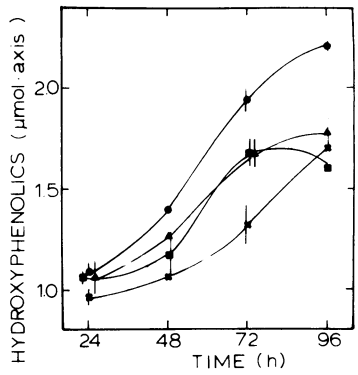

FIG. 3. Alcohol-soluble hydroxyphenolic compound contents of axes of soybean seedlings exposed to continuous white light and root-fed various chemicals after 3 days of dark growth. ●: Control; ■: 0.5 mM glyphosate; ▲: 0.1 mM AOPP; ×: 0.5 mM glyphosate and 0.1 mM AOPP.

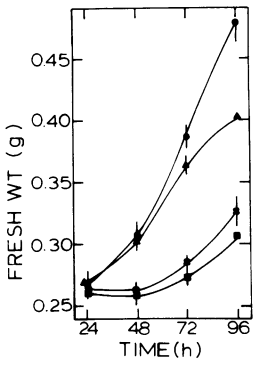

FIG. 1. Fresh weight gain of soybean axes from seedlings exposed to continuous white light and root-fed various chemicals after 3 days of dark growth. ●: Control; ■: 0.5 mM glyphosate; ▲: 0.1 mM AOPP; ×: 0.5 mM glyphosate and 0.1 mM AOPP.

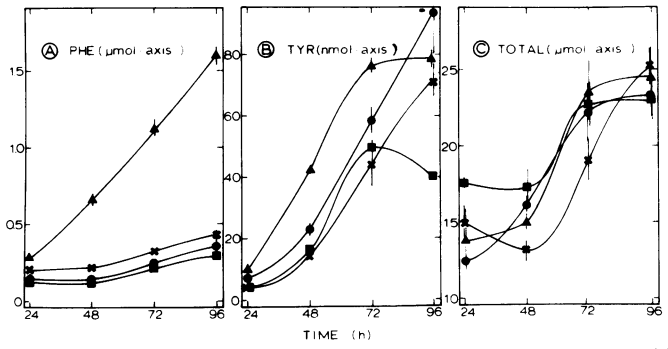

FIG. 4. Contents of phenylalanine (A), tyrosine (B) total amino acids (C) per axis of soybean seedlings exposed to continuous white light and root-fed various chemicals after 3 days of dark growth. ●: Control; ■: 0.5 mM glyphosate; ▲: 0.1 mM AOPP; ×: 0.5 mM glyphosate and 0.1 mM AOPP.

other than a reduction of total amino acids by glyphosate plus AOPP at 48 h (Fig. 4C).

Effects on Root and Shoot Separately. Generally, the effects of treatments on PAL activity were more pronounced in root than hypocotyl tissue after 72 h (Table I). In both organs the order of treatment effects on PAL activity was generally: AOPP < AOPP + glyphosate < glyphosate. AOPP had no effect on soluble protein, but glyphosate increased soluble protein concentration in both organs and decreased the amount per root as a consequence of fresh weight reduction. No significant effect of AOPP alone or with glyphosate on root or hypocotyl fresh weight was demonstrated. Most of the effects on hydroxyphenolics were in the root, where all three chemical treatments reduced the amount of these compounds per organ. AOPP alone was the only treatment which reduced the concentration (amount per g) of hydroxyphenolics.

Phenylalanine and tyrosine levels were significantly reduced in roots by glyphosate (Table II). AOPP caused a 7.2-fold increase in phenylalanine and a 1.4-fold increase in tyrosine in roots and 3.5- and 1.27-fold increases in phenylalanine and tyrosine in hypocotyls. The effect of glyphosate on phenylalanine content per organ was totally eliminated by AOPP in the roots, but the effect on tyrosine was not significantly changed. Twelve amino acids in the root and seven in the hypocotyl were affected by at least one of the treatments. Glyphosate effects on the levels of free amino acids in soybean roots and hypocotyls have been presented earlier (11, 17). AOPP increased levels of alanine and valine in the hypocotyl while increasing amounts of histidine in root tissue. Threonine, serine, glutamine, valine, isoleucine, and leucine were significantly reduced by AOPP in the roots. AOPP caused significant reversals of the glyphosate-induced proline increases in the hypocotyl and glycine reductions in the root.

DISCUSSION

Because the PAL inhibitor AOPP only marginally ameliorated the effects of glyphosate on growth, PAL is probably, in this case, only partially involved with glyphosate's mode of action. Our time course data for PAL activity suggest that glyphosate acts faster

Table I. *Physiological Parameters of Roots and Hypocotyls of 6-Day-Old Soybean Seedlings Exposed to Continuous White Light and Various Chemicals (Root-fed) for 3 Days*

The glyphosate and AOPP were present at 0.5 mM and 0.1 mM, respectively. Values in vertical columns under each organ followed by the same letters are not significantly different at the 95% confidence level. Numbers in parentheses are percentages of controls.

	PAL Activity (mU)			Soluble Protein (mg)		Fresh Weight (g)	Hydroxyphenolics (μmol)	
	mg protein^{-1}	g fresh weight^{-1}	Organ^{-1}	g fresh weight^{-1}	Organ^{-1}		g fresh weight^{-1}	Organ^{-1}
				Root				
Control	2.04a	9.60a	1.63a	6.50a	1.34a	0.179a	5.88a	1.00a
Glyphosate	5.25b (257)	30.5b (318)	3.11b (191)	9.58b (147)	0.97b (72)	0.101b (56)	6.31a (107)	0.64b (64)
AOPP	4.04c (198)	21.3c (222)	3.58b (220)	7.80ac (120)	1.41a (105)	0.165a (92)	4.52b (77)	0.72c (72)
AOPP + glyphosate	4.71bc (231)	25.7bc (268)	2.64b (162)	9.38bc (144)	0.87b (65)	0.098b (55)	6.30a (107)	0.59bc (59)
				Hypocotyl				
Control	1.37a	7.0a	1.63a	7.21a	1.63a	0.230a	5.31a	0.90a
Glyphosate	2.69b (196)	18.8b (269)	3.79b (233)	9.12b (126)	1.81a (111)	0.204b (89)	5.05ab (95)	1.04a (116)
AOPP	2.25b (164)	13.1c (187)	2.76c (169)	8.29a (115)	1.68a (103)	0.206b (90)	4.48b (84)	0.97a (108)
AOPP + glyphosate	2.53b (185)	19.2b (274)	3.64b (223)	10.18c (141)	1.89a (116)	0.190b (83)	4.68b (88)	0.90a (100)

Table II. *Free Amino Acid Profiles of Roots and Hypocotyls of Soybean Seedlings Exposed to Continuous Light and Root-fed Various Treatment Compounds for 3 Days after 3 Days of Dark Growth*

Within each line (one amino acid), numbers under each organ followed by the same letters are not significantly different at the 95% level. Glp = glyphosate.

Amino acid	Root				Hypocotyl			
	Control	AOPP	Glp	AOPP + Glp	Control	AOPP	Glp	AOPP + Glp
				nmol/organ				
Asp	50ab	47a	29bc	22c	76a	86a	81a	61a
Thr	236a	204b	106c	122c	320a	318a	347a	317a
Ser	356a	302b	175c	170c	474a	520a	559a	459a
Asn + Glu	3955a	4238a	3723a	3311a	12016a	12417a	13004a	10596a
Gln	50a	33b	60a	36ab	72a	80ab	161b	136b
Pro	73a	102a	120a	112a	60a	87a	262b	145c
Gly	54ac	73a	16b	34c	12a	11a	13a	11a
Ala	444a	475a	469a	353a	133a	221b	261b	202b
Val	442a	377b	190c	185c	682a	860b	1089b	855b
Ile	185a	153b	100c	90c	298a	331a	377a	288a
Leu	126a	108b	48c	41c	112a	128a	118ab	79b
Tyr	21a	30b	7c	10c	38a	47a	43a	34a
Phe	66a	477b	17c	66a	187a	652b	184a	255a
GABA	199a	228a	209ab	140b	163ab	195a	181ab	161b
Ethanolamine	51a	50a	24b	31b	24a	24a	9a	31a
NH_3	1261a	1229a	921b	938ab	1100a	1173a	1122a	1161a
Lys	19a	31a	30a	36a	31a	41ab	78ab	68b
His	305a	332b	237c	232c	255a	333a	361a	354a
Total minus NH_3	6819ab	7245a	5747ab	4991b	15191a	16337a	19125a	14052a

than AOPP. Pretreatment with AOPP before glyphosate treatment might have been more effective in preventing glyphosate effects by a build-up of aromatic amino acids (such as occurred in plants treated solely with AOPP). Compartmentalization poses another problem: we have no evidence that the effects on PAL activity, hydroxyphenolics, or amino acids caused by glyphosate or AOPP are in the same tissues, cells or intracellular compartments.

As a note added in proof Amrhein and Holländer (7) reported AOPP to increase levels of free phenylalanine 20- to 50-fold above normal in the hypocotyls of buckwheat seedlings after 24-h exposure. In our system there was little significant effect at 24 h, but thereafter both tyrosine and phenylalanine were significantly increased by AOPP. Tyrosine could have increased in two ways. First, the PAL of soybeans could also function to some degree as TAL. Previously we found a small amount of TAL activity associated with PAL activity in our preparations (11). Second, AOPP may have some inhibitory effect on the activity of a separate TAL enzyme. Our finding that glyphosate reduced AOPP-increased phenylalanine pools is supported by similar results in a recent abstract by Holländer and Amrhein (18). The slight effect of AOPP on the levels of other free amino acids indicated that it was ineffective as a general transaminase inhibitor at the level used.

A striking similarity was shown between the effects of glyphosate and AOPP on extractable PAL activity and soluble hydroxyphenolics. AOPP has been demonstrated to be the most potent *in vitro* PAL inhibitor known (5, 6). In our preparations, AOPP must have been removed from the enzyme during purification because of the high PAL activity measured. Our results further suggest *in vivo* inhibition of PAL activity, because total soluble hydroxyphenolic compounds were reduced while phenylalanine levels were increased. The enhanced levels of partially purified PAL caused by AOPP suggest that PAL end products either: (*a*) directly cause lower extractable activity by feedback inhibition due to incomplete removal during purification or; (*b*) are involved in end product repression of PAL synthesis. Two factors support the view that PAL synthesis is controlled by end products in this case: (*a*) the enzyme purification used eliminates a large proportion of the phenolic compounds of the crude extract; and (*b*) the increase in PAL specific acitivity caused by AOPP is continuous for at least 4 days. Evidence from others supports this explanation of AOPP-induced PAL increases. Amrhein's laboratory has recently found AOPP and AOA (a less potent and less specific PAL inhibitor [2, 6]) to cause superinduction of PAL activity in gherkin hypocotyls (3, 4). In earlier work they found no effect of AOPP on PAL from buckwheat hypocotyls in which end products were prevented from accumulating by AOPP (5). Similarly, glyphosate has no effect on extractable PAL levels of buckwheat seedlings (personal communication from N. Amrhein). The effects of AOPP or AOA in gherkin were eliminated by feeding cinnamic acid. Hydroxycinnamic acid accumulation was greatly reduced by AOPP and AOA in gherkin. Amrhein and Gerhardt (4) concluded that PAL reaction product(s) are involved to some extent in the regulation of the pool of PAL in gherkin tissue. Cinnamic and hydroxycinnamic acid regulation of extractable PAL activity in gherkin hypocotyls was hypothesized in earlier investigations (12, 13, 22). Szkutnicka and Lewak (27) found that exogenously supplied L-phenylalanine increased PAL levels, while D-phenylalanine (a competitive inhibitor of PAL) increased extractable PAL activity from seedlings of several species. Huault and Klein-Eude (19) had similar results with radish and also found that exogenously supplied *t*-cinnamic acid reduces extractable PAL activity.

Although the effects of glyphosate on extractable PAL activity appear to be similar to those of AOPP, glyphosate's effect may be through reduction of substrate levels, and thus reduced end product feedback inhibition of PAL activity or repression of PAL synthesis. New evidence of Holländer and Amrhein (18) indicates that glyphosate interferes with the shikimate pathway, thereby reducing aromatic amino acid levels. If this is the case, a fundamental difference between glyphosate and AOPP effects on PAL activity is that *in vivo* PAL activity is limited by lack of substrate by glyphosate and by enzyme inhibition in the case of AOPP. Previously we found no *in vitro* effect of glyphosate on PAL activity (10). The finding that AOPP partially reversed decreases in phenylalanine in glyphosate-treated plants suggests as one alternative that some of the depletion is due to increased levels of active PAL in glyphosate-treated tissues. However, that near normal levels of aromatic amino acids can be produced in glyphosate-treated plants with AOPP without greatly affecting glyphosate's effects on growth indicates that glyphosate's effects on aromatic compounds may not be its principal mode of action. Some evidence exists that glyphosate may act as a respiratory inhibitor (1) through uncoupling mitochondrial phosphorylation (24) at high concentrations.

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