MODE OF SAFENING ACTION OF NAPHTHALIC ANHYDRIDE AGAINST INJURY OF SULFONYLUREA AND IMIDAZOLINONE HERBICIDES IN MAIZE

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Summary. Safening action of naphthalic anhydride (NA) in maize, *Zea mays*, treated with sulfonylurea and imidazolinone herbicides and effect of NA and the herbicides on *in vitro* acetolactate synthase (ALS), glutathione S-transferase (GST), and acetyl Co-A carboxylase (ACCase) activities were investigated. NA provided safening factors of approximately 11 and 10 for chlorsulfuron and bensulfuron, respectively, and 5 and 4 for imazaquin and imazethapyr, respectively. NA caused 1.3-, 1.8-, and 3.2-fold increase of ALS, GST, and ACCase activities, respectively. The activities of ALS extracted from both NA-treated and NA-untreated maize were inhibited by bensulfuron and imazaquin. However, the two herbicides did not affect the activities of ACCase obtained from both NA-treated and NA-untreated maize, except for bensulfuron on NA-induced ACCase activity. NA-induced ACCase activity slightly increased with increase in bensulfuron concentration. These results suggested that the safening mode of action of NA might result from multilevel interaction of NA-induced enzyme activities.

INTRODUCTION

The sulfonylurea and imidazolinone herbicides are new families of herbicide introduced in the early 1980s which are highly active at low application rates. The sulfonylureas control a wide range of annual broad-leaved and certain grass weeds in small grain cereals, while the imidazolinones are used for control of annual and perennial grasses and broad-leaved weeds. They can enter plants through roots and foliage and have the same site of action, that is, they inhibit ALS the first enzyme in the synthetic pathways for branched-chain amino acids (9, 10).

Many researches have shown that herbicide safener NA has potential in protecting grass crops from toxicity caused by these herbicides (1, 8). Parker *et al.* (8) demonstrated a reduction in chlorsulfuron activity on maize following NA treatment. Protection of maize against injury from pre-emergence imazaquin application was also achieved by NA treatment of seeds (1).

In a review of mechanisms of safener action, Hatzios (7) indicated that the possible mechanism which receives the most attention is either a safener-induced enhancement of herbicide detoxication or a competitive antagonism of herbicides and safeners at a common target site of action. Based on the suggestion, this study was initiated to evaluate effects of NA on activities of three enzymes related with crop-herbicide-safener combination. The enzymes included were ALS as target site of both sulfonylurea and imidazolinone herbicide action and GST involved in the detoxication of certain herbicides. ACCase was also included since it is a key enzyme in the pathway of fatty acid biosynthesis and the target site of graminaceous herbicides (3, 6).

METHODS

Safening effect of NA. Before planting dressing of maize (cv. 'Suweon 19') seeds with NA was done by shaking in a flask at rate of 0.2% by seed weight. Fifteen seeds of the maize were planted approximately 2 cm deep in a sterilized clay loam soil in 350 cm^2 plastic pots. There were three replications. One day after seeding four herbicides (bensulfuron, chlorsulfuron,

imazaquin, and imazethapyr) were soil-applied at rates of 6.25, 12.5, 25, 50, 100, and 200 g/ha. After planting and treatment with the herbicides, the pots were placed in a greenhouse with a 14-h photoperiod and a 30/20°C day/night temperature. All pots were watered from overhead to the soil surface as required. The plants were harvested 21 days after herbicide treatment to measure the fresh weights. On the basis of the results obtained, concentrations of the herbicides causing 50% inhibition (I_{50}) were calculated by probit analysis. Safening factor of NA was then calculated by the ratio of the herbicide concentration giving I_{50} in the presence of NA divided by the herbicide concentration giving I_{50} in the absence of NA (4).

Plant material for enzyme assay. Shoots of 10-day old maize seedlings grown in the conditions as described above were used for ALS and ACCase assays. To obtain etiolated shoots for extraction of GST, maize treated with NA was planted in vermiculite, watered, maintained in a growth chamber in the dark for 3 days at 30°C.

ALS assay. All steps were performed at 4°C unless otherwise noted. Fifty g of the shoots was ground in a mortar prechilled in liquid nitrogen and homogenized in 100 mL of buffer containing 0.1 M K_2HPO_4 (pH 7.5), 5 mM $MgCl_2$ and 10 mM sodium pyruvate. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 15,000 *g* for 15 min. ALS was precipitated from the supernatant fluid with $(NH_4)_2SO_4$. The enzyme was collected at 65% saturation by centrifugation and the pellet dissolved in the buffer as described above and desalted on Sephadex G-25 (PD-10) equilibrated with the same buffer. The desalted enzyme was used immediately for assay. ALS assay was conducted in a final volume of 2 mL at 30°C. The final reaction mixture consisted of 1 mL of the desalted enzyme, 0.9 mL of 50 mM K_2HPO_4 (pH 7.5) containing 10 mM sodium pyruvate, 0.1 mM thiamine pyrophosphate and 10 mM $MgCl_2$ and 0.1 mL of various concentrations of the herbicides dissolved in acetone. Assay was initiated by adding the pyruvate and terminated by adding 30 µl of 10 N H_2SO_4. ALS activity was determined as described by Westerfeld (11). The acidified reaction mixtures were heated for 15 min at 60°C after which 0.5 mL of 0.5% w/v creatine and 0.5 mL of 5% w/v 1-naphtol dissolved in 10% NaOH were added. The solution was heated for an additional 15 min at 60°C. The absorbance of the solution was then determined at 530 nm. Protein was determined by the method of Bradford (2).

GST assay. Five g of the shoots of etiolated maize seedlings was ground in liquid nitrogen and homogenized in 25 mL of buffer containing 0.1 M K_2HPO_4 (pH 6.8) and 1 mM sodium metabisulfite. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 20,000 *g* for 20 min. The supernatant was used immediately for assay. GST assay was carried out in a final volume of 2 mL. The reaction mixture contained 0.1 mL of the enzyme extract, 1.9 mL of 100 mM K_2HPO_4 (pH 7.5) and 0.9 mL of 3.3 mM glutathione (reduced form). Assay was initiated by adding 0.1 mL of 30 mM 1-chloro-2,4-dinitrobenzene as the substrate at 25°C. GST activity was measured spectrometrycally at 340 nm for 2 min starting from 1 min after initiation of the reaction.

ACCase assay. Ten g of the plant material was ground in 20 mL of cold extraction buffer containing 0.1 M tricine-KOH (pH 8.0), 10 mM b-mercaptoethanol, 1 mM Na-EDTA and 1 mM phenylmethylsulfonylfluoride. The plant slurry was filtered through 8 layers of cheesecloth and the filtrate was centrifuged at 30,000 *g* for 20 min. The supernatant was used directly for assay. ACCase activity was assayed at 35°C in a 0.2 mL volume which contained 20 mM ATP, 3 mM acetyl CoA, 50 mM $MgCl_2$, 20 mM DTT, 20 mM $NaH^{14}CO_3$ (20 µCi/mmol) and the herbicides. Reactions were initiated by addition of acetyl CoA and stopped by addition of 30 µl of 12 N

HCl. Product formation was determined by the radioactivity found in an acid stable fraction by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Effect of NA on herbicidal activity. Herbicidal activity of the two groups of herbicides as measured by I_{50} varied with kind of the herbicides used (Table 1). The lowest I_{50} was obtained with chlorsulfuron, whereas I_{50} of bensulfuron and imazethapyr was about 8-fold higher than that of chlorsulfuron. However, no clear difference in I_{50} was found between the two groups. With use of NA I_{50} of the four herbicides increased, indicating safening effect of NA. The safening effect was quantified by safening factor. NA provided safening factors of approximately 10 and 11 for bensulfuron and chlorsulfuron, respectively, and 5 and 4 for imazaquin and imazethapyr, respectively. The safening effect due to NA differed between the two groups of herbicides, but no great difference occurred between the herbicides within the same group. Efficacy of NA in protecting maize from injury of sulfonylurea and imidazolinone herbicides has been reported also by other investigators (1, 8).

Table 1. Herbicide concentration of 50% inhibition and safening factor of combined treatments of NA and sulfonylurea and imidazolinone herbicides on maize

Herbicide	50% Inhibitation (g/ha)		Safening factor
	Without NA	With NA	
Bensulfuron	29.1	296.7	10.2
Chlorsulfuron	3.8	42.6	11.2
Imazaquin	7.8	39.5	5.1
Imazathapyr	29.1	116.1	4.0

Elevation of NA-induced enzyme activity. ALS, GST and ACCase activities increased with treatment of NA in maize (Table 2). The greatest increase occurred with ACCase, while the least with ALS. The increases resulted from NA-induced enzyme production. However, elevation of any of the enzyme activities by NA did not exactly correspond to the safening factors. This indicated that effect of NA on the enzyme activation or enzyme induction process had no simple correlation with the protective action.

Table 2. Effect of NA on elevation of enzyme activity in maize

Enzyme	Activity (uM/min/mg protein)		
	Without NA (A)	With NA (B)	B/A
ALS	6.9	9.1	1.3
GST	0.9	1.6	1.8
ACCase	0.025	0.079	3.2

Effect of herbicides on NA-induced enzyme activity. ALS activity decreased linearly with logarithmic increase in bensulfuron concentration starting from 10^{-9}M, while the same effect

occurred with imazaquin at 10^{-6}M (Fig. 1). A similar trend was found in NA-induced ALS activity. With both herbicides tested, however, there was difference in inhibition of ALS activity between ALS's obtained from NA-treated and NA-untreated maize. The inhibition difference decreased with increase in herbicide concentration. This difference is due probably either to NA-induced altered ALS forms which are less sensitive to the herbicide inhibition or to *de novo* synthesis of ALS caused by NA, or both.

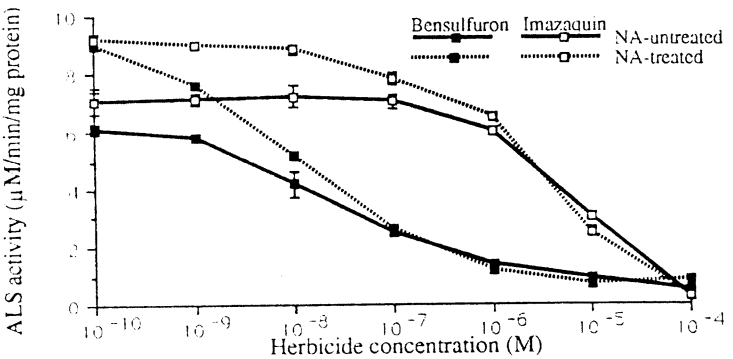

Figure 1. Effect of bensulfuron and imazaquin on NA-induced ALS activity of maize.

Effect of bensulfuron and imazaquin on ACCase activity varied with the source of the enzyme preparation (Fig. 2). Activity of ACCase obtained from NA-untreated maize was not affected by concentration of the two herbicides up to 10^{-5}M. On the other hand, a slight increase in activity of ACCase obtained from NA-treated maize occurred by bensulfuron concentration between 10^{-8} and 10^{-5}M, while there was no significant difference in ACCase activity with imazaquin at the same concentration range. Increase in ACCase activity by bensulfuron might be result of activation of NA-induced ACCase. However, the different response between the herbicide groups was possibly attributed to different sensitivity of the enzyme to the herbicides.

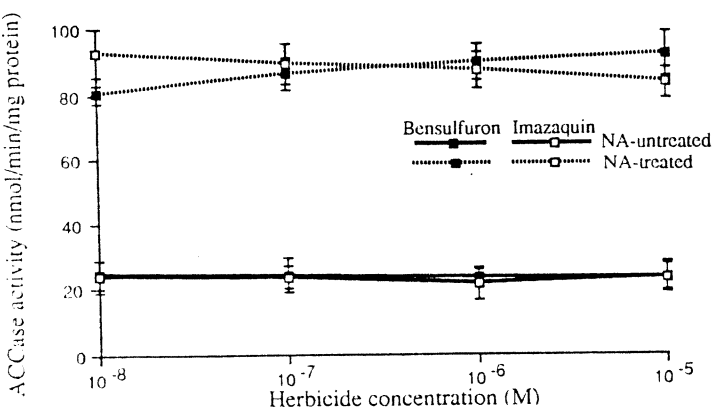

Figure 2. Effect of bensulfuron and imazaquin on NA-induced ACCase activity of maize.

The results presented in this paper demonstrate that NA provided a significant protective action against injury of sulfonylurea and imidazolinone herbicides to maize. The degree of the safening effect, however, varied with the herbicide classes. Safening factors on sulfonylureas were two-fold or greater than those on imidazolinones. This difference may be due either to differential sensitivity of maize to the herbicide classes or to different specificity of NA-herbicide combinations.

In the safening mode of action of NA, NA-induced GST activity may not play an important role. Although NA increased GST activity by about 80%, the NA-induced GST would not act in the detoxication of the herbicides through glutathione conjugation. Frear *et al.* (5) found that NA increased 40-60% GST activity in maize, whereas chlorsulfuron did not alter glutathione content and GST activity. On the other hand, about 30% increase in extractable ALS was also caused by NA. However, the increase appears minor in comparison to the degree of the safening. This fact suggests that competitive antagonistic action of the two herbicide classes and NA on ALS may be excluded in elucidating the potential safening mechanism of action of NA.

The greatest increase in NA-induced enzyme activity studied occurred with ACCase. ACCase catalyzes the first committed step in fatty acid biosynthesis (6) and has been identified as the target site involved in the phytotoxic action of two classes of graminaceous herbicides, the aryloxy-phenoxypropionate and the cyclohexanedione (3). Although increase in ACCase activity by NA is not of sufficient magnitude to account for the safening activity of NA on maize, involvement of ACCase activity in protective action of NA against injury of sulfonylurea and imidazolinone herbicides in maize is rather of interest. This enzyme is not a target site for either sulfonylurea and imidazolinone herbicides or NA. According to the finding of Yenne and Hatzios (12), activity of ACCase extracted from oxime ether safener-treated sorghum, *Sorghum bicolor*, did not differ from that extracted from untreated sorghum. Therefore, it is more likely that NA-induced ACCase may be consequence of NA-maize specificity.

Based on the results obtained, it is reasonable to hypothesize that safening mode of action of NA results from multilevel interaction of enzymes responsible for crop-herbicide-safener combination. Any of the NA-induced enzymes alone could not provide sufficient increase in the enzyme activity to explain the degree of the safening effect of NA. Activities of NA-induced ALS, GST, and ACCase might be required at the same time to exert the protective action of NA against injury of sulfonylurea and imidazolinone herbicides in maize.

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