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RESEARCH PAPER

Characterization of aryl acylamidase activity from propanil-resistant barnyardgrass (*Echinochloa crus-galli* [L.] Beauv.)

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The enzyme, aryl acylamidase, was characterized in propanil-susceptible and propanil-resistant barnyardgrass with respect to kinetic parameters, the effects of inhibitors, and the levels of activity in dark- and light-grown tissues. The enzyme reaction in the resistant tissue preparation proceeded linearly with time over a 5 h time course, while activity in the susceptible tissue preparation was 2- to 4-fold lower and the activity tended to decrease after 2 h. The apparent K_m values were 62.1 mmol L^{-1} and 3.1 mmol L^{-1} for the enzyme activity in the susceptible and resistant tissue preparations, respectively. Two herbicides (anilofos and piperophos), previously shown to synergize propanil injury against the resistant biotype, were found to be potent inhibitors of the *in vitro* aryl acylamidase activity.

Keywords: aryl acylamidase, barnyardgrass, propanil, resistance.

INTRODUCTION

There are several *Echinochloa* grass species, but two (barnyardgrass and junglerice, *Echinochloa crus-galli* [L.] Beauv. and *Echinochloa colona* [L.] Link, respectively) are especially troublesome weeds. Barnyardgrass has had the distinction of being the world's worst weed in rice (Holm *et al.* 1977). This weed can cause ≤ 75% reduction in rice grain yield (Carey 1994) and a barnyardgrass density as low as 1 plant m^{-2} can reduce grain yield (Stauber *et al.* 1991). Propanil, an acylanilide herbicide synthesized by Rohm and Haas in 1957 (Eberlein 1990), was introduced into cultivated rice (*Oryza sativa* L.) production in the USA in 1962 for broad-spectrum, postemergence control of dicotyledonous and monocotyledonous weeds, including *Echinochloa* spp. (Smith 1961). Propanil has been used extensively in rice production in the USA in all rice-producing states and in several other countries, but is no longer labeled for use in California. The molecular mode of action of propanil is inhibition of photosystem II (PSII), but rice is tolerant to propanil due to the presence of high levels of the enzyme, aryl acylamidase, that catalytically degrades the molecule to non-phytotoxic compounds, that is, 3,4,-dichloroaniline and propionic acid (Frear & Still 1968; Akatsuka 1979).

As recently as ≈ 10 years ago, various populations of barnyardgrass were discovered to exhibit resistance to propanil applied at the label-recommended use rates of 3.6–5.6 kg ai ha^{-1}. In Arkansas, this resistance was reported in several geographic areas (Carey *et al.* 1992; Baltazar & Smith 1994). Likewise, in other rice-producing countries, certain barnyardgrass biotypes began to exhibit resistance to propanil (Garro *et al.* 1991; Fischer *et al.* 1993). In Texas, 11 samples suspected of being propanil-resistant barnyardgrass were actually found to be a closely related weed, junglerice (Carey *et al.* 1995b). Of these junglerice samples, four were found to be susceptible and seven were resistant to propanil (Carey *et al.* 1995b). Propanil-resistant junglerice also has been verified in rice-producing areas of Greece (Giannopolitis & Vassiliou 1989), Columbia (Fischer *et al.* 1993), Costa Rica (Garro *et al.* 1991), El Salvador, Guatemala, Nicaragua, Panama, and Mexico (Villa-Casarez 1998).

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Shortly after the discovery of propanil resistance in barnyardgrass and junglerice, studies were initiated to ascertain their resistance mechanisms. In studies on a propanil-resistant barnyardgrass biotype from Arkansas, differential propanil uptake and/or translocation, cross-resistance to other PSII-inhibiting herbicides, and a modified propanil site of action were shown not to be involved in the resistance mechanism (Carey *et al.* 1995a). However, *in vivo* metabolism studies on this biotype showed that elevated metabolism was responsible for this resistance (Carey *et al.* 1997). Similar results were obtained in propanil-resistant junglerice (Lopez-Martinez *et al.* 2001). Research on two propanil-resistant biotypes of junglerice demonstrated that the resistance mechanism was also elevated aryl acylamidase activity (Leah *et al.* 1994, 1997). In propanil-resistant junglerice, the aryl acylamidase was isolated and some of its properties elucidated (Leah *et al.* 1997). However, there have been no reports on the isolation and characterization of this enzyme from propanil-resistant barnyardgrass. We now report the isolation of this enzyme, the determination of some of its properties, and some comparisons with this enzyme from rice and propanil-susceptible barnyardgrass.

MATERIALS AND METHODS

Plant source and growth conditions

Seeds of propanil-susceptible and propanil–resistant barnyardgrass (*Echinochloa crus-galli* L.) were obtained from verified samples from the University of Arkansas (Carey *et al.* 1997). The seeds of the barnyardgrass and rice (*Oryza sativa* L. cv. Lemont; MAFES Foundation Seed Stocks, Mississippi State, MS, USA) were planted in vermiculite, watered with dilute nutrient solution, grown for 6 days under darkness at 25°C, and then harvested for extraction. Some tests were performed using plants grown similarly in a growth chamber under light (200 $\mu E\ m^{-2}\ s^{-1}$).

Enzyme extraction

The leaves of 6 day old etiolated barnyardgrass or rice were excised at the vermiculite surface and homogenized in a chilled phosphate buffer (100 mmol L^{-1}, pH = 7.5, containing 1 mmol L^{-1} dithiothreitol and 2% insoluble polyvinylpyrollidone) using a chilled mortar and pestle on an ice block. The ratio of the leaf sample amount to the buffer volume was generally 1 g fresh weight to 10 mL. The homogenate was then centrifuged at 2000 *g* for 5 min and the supernatant was used directly as the crude enzyme preparation.

Enzyme assay

The standard reaction mixture contained 940 μL enzyme solution, 10 μL water, and 50 μL propanil in dimethyl sulfoxide (20 mmol L^{-1}) to give a final propanil concentration of 1 mM. The reactions were typically run for 3 h at 30°C in a water bath. The reactions were terminated by the addition of 500 μL of 20% trichloroacetic acid (TCA). Denatured protein was removed by centrifugation (2000 *g*, 5 min). Fifteen minutes after the addition of 1500 μL of *p*-dimethylaminocinnamaldehyde (DACA; 0.12%, dissolved in ethanol) to 1000 μL of the TCA supernatant, the product of the enzyme reaction, 3,4-dichloroaniline (DCA), was quantified by measuring the absorbance at A_{540} nm. The enzyme reaction proceeded linearly with the protein concentration < 0.5 mg mL^{-1} in barnyardgrass and < 0.2 mg mL^{-1} in rice, respectively. The specific activity was calculated as one unit of activity = 1 nmol DCA produced min^{-1} mg^{-1} of protein. All enzyme assays in this study were conducted with three replications.

Enzyme inhibitor tests

Compounds tested for enzyme inhibition were: anilophos, *S*-[2-[(4-chlorophenyl)(1-methylethyl)amino]-2-oxoethyl] *O,O*-dimethylphosphorodithioate; piperophos, *S*-[2-(2-methyl-1-piperidinyl)-2-oxoethyl] *O,O*-dipropyl phosphorodithioate; PPG-124, *p*-chlorophenyl-*N*-methycarbamate; and carbaryl, 1-naphthyl *N*-methycarbamate. They were prepared in dimethyl sulfoxide and aliquots of these preparations were incorporated into the standard assay mixture and were assayed in 3 h reaction times. The final concentrations of the inhibitor test compounds in reaction assay mixtures ranged from 20–200 μmol L^{-1}. All compounds were ≥ 98% in purity.

Kinetic measurements

Kinetic analyses of the enzyme using propanil as the substrate in propanil-resistant and propanil-susceptible barnyardgrass enzyme preparations were performed at 30°C. Various amounts of the propanil substrate stock were incorporated into the standard reaction mixture. The data was plotted using typical double reciprocal plots, that is, reciprocal of velocity versus reciprocal of substrate concentration.

Protein determination

The protein concentration in all aliquots was determined colorimetrically using the Bradford reagent

(Bradford 1976). Bovine serum albumin was used as the protein standard.

Chemical sources

All chemicals used in this study were of reagent grade or higher in purity. Standard reagents for enzymological study were purchased from Sigma Chemical Company, St Louis, MO, USA. High-purity herbicides and insecticides were purchased from Chem Service, West Chester, PA, USA.

RESULTS AND DISCUSSION

Aryl acylamidase detection and standard assay

The detection of aryl acylamidase from the propanil-resistant barnyardgrass was facilitated using a dichloroaniline coupling agent that provided greater sensitivity than that using the diazotization method (Bratton & Marshall 1939). This coupling agent, DACA, was more sensitive and more rapid than the older, multistepped diazotization method used in many previous studies of aryl acylamidase (e.g. Frear & Still 1968; Hoagland & Graf 1972; Hoagland 1975, 1978). In tests to optimize the standard assay, we found that a 30°C reaction temperature provided greater activity and reduced incubation times when compared to lower reaction temperatures or to temperatures of ≥ 35°C (data not shown). Therefore, a 30°C temperature was chosen for the standard assay.

These results are consistent with other reports on aryl acylamidases from other sources. Injury to rice, as well as increased barnyardgrass control, often occur at temperatures of ≥ 35°C. This may be attributed to greater propanil absorption and reduced aryl acylamidase activity, subsequently leading to reduced propanil metabolism at higher temperatures. For instance, Hoagland (1978) reported that *in vitro* aryl acylamidase activity from red rice (*Oryza sativa* L.) decreased at exposure temperatures > 35°C. Wild rices (*Oryza* spp.) also have greater aryl acylamidase activity when grown at 20–25°C than at 30°C (Jun & Matsunaka 1990).

Aryl acylamidase activity in light- and dark-grown seedlings

The enzyme activity in preparations from etiolated tissues was generally higher (10–12%) than in preparations from light-grown plants (data not shown); thus, most tests utilized the dark-grown tissue for the enzyme preparations used in this study. The comparison of aryl acylamidase activity from the tissues of etiolated rice and the resistant and susceptible barnyardgrass biotypes indicated that the activity in rice was 1.7-fold higher than in the resistant biotype and 6-fold higher than in the susceptible biotype (Table 1). Aryl acylamidase activity in propanil-resistant barnyardgrass was linear over a 5 h assay time course, but the activity of the susceptible preparation was linear for only about 2 h and then it leveled off (Fig. 1). Furthermore, the activity in the resistant preparation was substantially higher than in the case of the susceptible biotype preparation. Overall, the activity in the resistant biotype was 2- to 4-fold higher compared to that in the susceptible biotype. The enzyme activity, as affected by the substrate (propanil) concentration in

Table 1. Comparison of aryl acylamidase activity in the leaves of etiolated rice and barnyardgrass biotypes†

Tissue	Specific activity ± standard error (nmol DCA min^{-1} mg^{-1} of protein)
Rice	2.44 ± 0.02
Resistant barnyardgrass	1.46 ± 0.02
Susceptible barnyardgrass	0.41 ± 0.01

† Enzyme was extracted from etiolated leaf tissues and assayed according to the standard assay as described in the Materials and Methods section. DCA, 3,4-dichloroaniline.

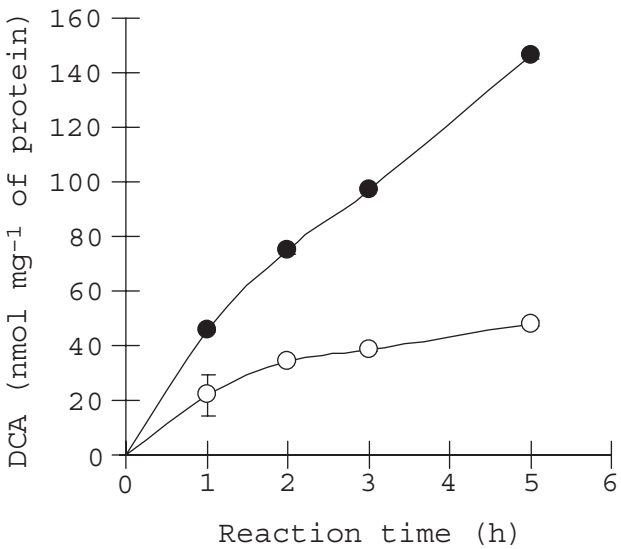

Fig. 1. Comparison of aryl acylamidase activity from etiolated seedlings between propanil-resistant (●) and propanil-susceptible (○) barnyardgrass biotypes over a 5 h time course using the standard assay conditions as described in the Materials and Methods section. The bars represent ± standard error. DCA, 3,4-dichloroaniline.

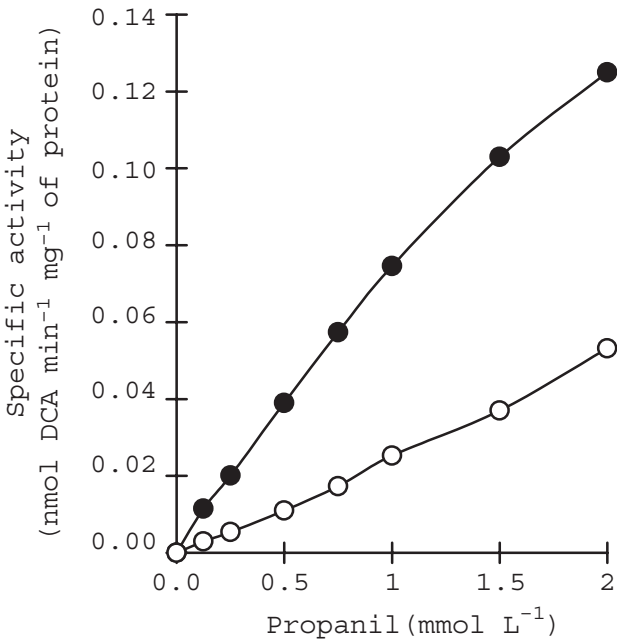

Fig. 2. Relationship between propanil concentration and the specific activity of aryl acylamidase activity in the etiolated leaves of propanil-resistant (●) and propanil-susceptible (○) barnyardgrass. The bars representing ± standard error are hidden behind the symbols. DCA, 3,4-dichloroaniline.

preparations from these two biotypes, was also compared (Fig. 2). The activity in the propanil-resistant biotype was substantially greater than that of the susceptible biotype at low, up to very high, propanil concentrations, which indicates major differences in the two enzymes. These results substantiate the findings of *in vivo* metabolic studies using ^{14}C (carbon)-labeled propanil in these barnyardgrass biotypes (Carey *et al*. 1997). This data is more proof that the mechanism of resistance in propanil-resistant barnyardgrass is indeed elevated propanil metabolism in the resistant biotype (Carey *et al*. 1997; Hoagland *et al*. 1997). Thus, the metabolic pathway in propanil-resistant barnyardgrass might be similar to that in rice, as elucidated in other reports (Frear & Still 1968; Still 1968; Yih *et al*. 1968): propanil is hydrolyzed to DCA, which is then conjugated with glucose and/or other saccharides. The mechanisms of herbicide resistance in weeds are generally different to the selectivity mechanisms in the crops on which the herbicides are used (LeBaron & McFarland 1988). In propanil-resistant barnyardgrass, however, the mechanism of resistance is the same as that found in rice. This also has been shown for another *Echinochloa* weed species that occurs in rice, junglerice (Leah *et al*. 1994).

In these present studies, very young plants were used for the enzyme preparations. The activity of this enzyme does vary depending on plant age and other factors in some species. Aryl acylamidase activity in propanil-resistant junglerice was higher than that in the susceptible biotype at all growth stages. Fischer *et al*. (1996) reported plant extracts from 2- to 3-leaf propanil-resistant junglerice had higher levels of propanil metabolism than the susceptible biotype. A direct correlation between elevated aryl acylamidase activity and propanil resistance in junglerice has been demonstrated (Leah *et al*. 1994). The specific activity of this enzyme using propanil as the substrate was 3-fold higher in the resistant junglerice biotype compared to the susceptible junglerice biotype and was ≈ 80% of the value found for rice enzyme preparations. Both the total and specific enzyme activity increased with junglerice plant age up to 15 days (4-leaf stage), then decreased after 20 days to about half of the maximum at 36 days. Biochemical analysis of partially purified aryl acylamidases from junglerice and rice seedlings indicated that these enzymes possessed similar pH optima (pH = 7.5) and native molecular masses, as estimated by gel filtration (Leah *et al*. 1997).

Aryl acylamidase inhibitor tests

Results from various reports have shown that synergistic or additive interactions of propanil occur with several compounds such as anilofos, piperophos, PPG-124, and others (Hoagland *et al*. 1999; Norsworthy *et al*. 1999a,b). PPG-124, which lacks insecticidal or herbicidal activity, has been commercialized as a herbicide synergist for amide herbicides, including propanil (Anonymous 1983). PPG-124 and the insecticide, meththiocarb, gave potent synergistic responses in resistant barnyardgrass as measured by laboratory bioassay methods, that is, chlorophyll fluorescence analysis of PSII inhibition (Hoagland *et al*. 1999) and reduction of total chlorophyll content (Hoagland *et al*. 1999). In the present study, some of these chemicals were tested for inhibitory effects on the *in vitro* activity of aryl acylamidase from etiolated tissues (Table 2). The two herbicides, anilofos and piperophos, inhibited the activity in the resistant biotype to a greater extent than in the susceptible biotype. Anilophos and piperophos exhibited a 47% and 44% inhibition, respectively, in the resistant biotype, but gave only a 20–22% inhibition in the susceptible preparation. This evidence is the first demonstration that these two herbicides are actual *in vitro* aryl acylamidase inhibitors. This also explains their synergistic effect when combined with propanil (Hoagland *et al*. 1999; Norsworthy *et al*. 1999a,b). PPG-124 was the most

Table 2. Effects of various compounds on aryl acylamidase activity in propanil-resistant and propanil-susceptible barnyardgrass from etiolated leaf tissue†

Barnyardgrass biotype	Compound	Concentration (μmol L^{-1})	% inhibition‡ ± standard error
Resistant	Anilofos	200	47.4 ± 1.3
	Piperophos	200	44.4 ± 1.5
	PPG-124	20	72.6 ± 1.0
Susceptible	Anilofos	200	22.1 ± 4.9
	Piperophos	200	20.5 ± 6.5
	PPG-124	20	63.9 ± 1.7

† Enzyme was extracted from etiolated leaf tissues and assayed according to the standard assay as described in the Materials and Methods section; ‡ data were presented as a percentage of the control at 100%, that is, a standard assay mixture with no inhibitor. The absolute values of the control were 0.77 nmol DCA min^{-1} mg^{-1} of protein for the resistant biotype and 0.31 nmol DCA min^{-1} mg^{-1} of protein for the susceptible biotype, respectively. DCA, 3,4-dichloroaniline.

Table 3. Effects of various compounds on aryl acylamidase activity in propanil-resistant and propanil-susceptible barnyardgrass from light-grown seedlings†

Barnyardgrass biotype	Compound	Concentration (μmol L^{-1})	% inhibition‡ ± standard error
Resistant	Anilofos	200	73.6 ± 1.0
	Piperophos	200	72.5 ± 1.3
	PPG-124	20	76.5 ± 0.8
	Carbaryl	200	81.6 ± 0.1
Susceptible	Anilofos	200	25.8 ± 1.7
	Piperophos	200	29.5 ± 4.8
	PPG-124	20	24.9 ± 1.8
	Carbaryl	200	56.8 ± 0.5

† Enzyme was extracted from light-grown leaf tissues and assayed according to the standard assay as described in the Materials and Methods section; ‡ data were presented as a percentage of the control at 100%, that is, a standard assay mixture with no inhibitor. The absolute values of the control were 0.055 nmol DCA min^{-1} mg^{-1} of protein for the resistant biotype and 0.011 nmol DCA min^{-1} mg^{-1} of protein for the susceptible biotype, respectively. DCA, 3,4-dichloroaniline.

potent inhibitor and, at a concentration of one-tenth that of the other inhibitors tested, inhibited by 73% and 64% in the resistant and susceptible preparations, respectively. In the enzyme preparations from light-grown tissue from the two barnyardgrass biotypes using the three compounds above and the insecticide, carbaryl, the two herbicides had somewhat different potencies when the effects on the resistant versus the susceptible preparations were compared (Table 3). Furthermore, the most dramatic difference in the effects on the etiolated tissue and light-grown tissue activity was caused by PPG-124. The inhibition ratio of the etiolated : light preparations for PPG-124 was 2.6:1 in the susceptible biotype. Anilofos and piperophos were also potent *in vitro* inhibitors of the enzyme from light-grown seedlings. Strong inhibition was also exhibited by the insecticide, carbaryl, and the inhibition was 1.4-fold greater on the resistant barnyardgrass than in the susceptible preparation from light-grown seedlings. Carbaryl has been known as a potent *in vivo* and *in vitro* aryl acylamidase inhibitor for some time (Bowling & Hudgins 1966; Frear & Still 1968; Yih *et al.* 1968; Hoagland 1975) and other organophosphate and carbamate insecticides inhibit aryl acylamidase in rice (Khodayari *et al.* 1986; Matsunaka 1968; Smith & Tugwell 1975). Propanil plus carbaryl combinations were injurious to rice (Norsworthy *et al.* 1999a), but rice injury as high as 56% at 13 days after treatment caused by 3.3 kg ha^{-1} propanil plus 0.33 kg ha^{-1} carbaryl had no adverse effects on rice yield (Talbert *et al.* 1996). Carbaryl and parathion-methyl also synergised propanil injury in propanil-resistant junglerice seedlings (Caseley & Leah 1996). Furthermore, selectivity in rice seedlings and synergy against propanil-resistant junglerice were achieved by applying a mixture of propanil with piperophos. The treatment of junglerice leaves with aryl acylamidase inhibitors, such as carbaryl or piperophos

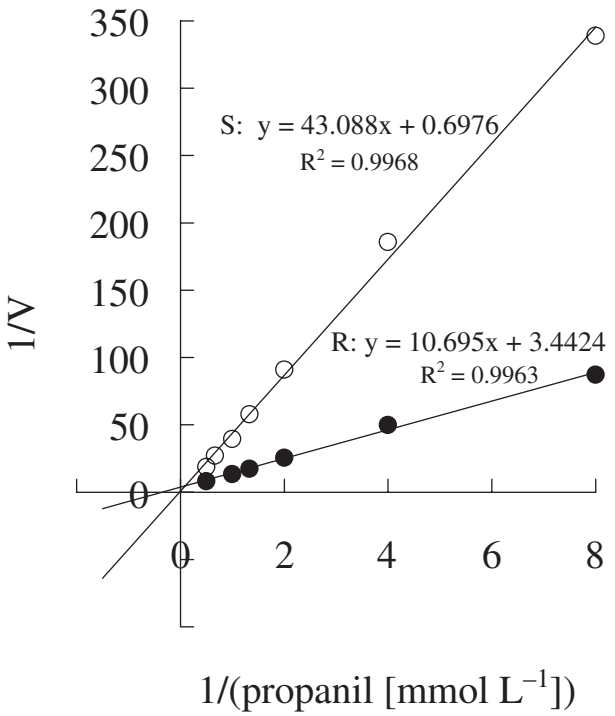

Fig. 3. Kinetic analysis of aryl acylamidase activity from propanil-resistant (●) and propanil-susceptible (○) barnyardgrass biotypes.

combined with propanil, significantly reduced resistance in plants at the growth stage, where aryl acylamidase activity was maximal. (Leah *et al.* 1995). Carbamate and organophosphorus pesticides were inhibitory to enzyme activity in these partially purified rice and junglerice preparations (Leah *et al.* 1994). Piperophos, when combined with propanil, provided control of propanil-resistant junglerice and increased rice yields (Valverde 1996; Valverde *et al.* 2001).

Some data using aryl acylamidase inhibitors *in vitro* indicated a slightly greater inhibition by carbamate and organophosphorous insecticides in junglerice aryl acylamidase compared to rice, which might be related to differences in enzyme kinetic parameters (Leah *et al.* 1994). Attempts to synergize or increase the control of propanil-resistant barnyardgrass with aryl acylamidase inhibitors and other chemicals using whole-plant screening in the field and greenhouse were carried out (Kitt 1995; Hoagland *et al.* 1999; Norsworthy *et al.* 1999a,b).

Kinetic tests of aryl acylamidase

When the aryl acylamidases from propanil-susceptible and propanil-resistant barnyardgrass were assayed at various propanil concentrations, the kinetics were dramatically different (Fig. 3). The apparent Km values were 62.1 mmol L^{-1} and 3.1 mmol L^{-1} for the enzyme in the susceptible and resistant biotypes, respectively.

Kinetic analysis reportedly showed that the junglerice enzyme had a lower affinity for propanil than the rice enzyme. Partially purified aryl acylamidase from rice has a 3-fold higher affinity for propanil compared to the enzyme in propanil-resistant and propanil-susceptible junglerice (Leah *et al.* 1995). The activity of these enzymes on several substrates showed the same relative order of substrate preference in rice, sensitive junglerice, and resistant junglerice. The relative rates of activity on each substrate were: rice > resistant junglerice > susceptible junglerice (Leah *et al.* 1994).

Comparative studies on aryl acylamidases from propanil-resistant barnyardgrass will aid in establishing the important differences in these proteins from other sources, including propanil-resistant junglerice. Biochemical research is needed to discover other potent synergists, to isolate, purify, and characterize the aryl acylamidase in the resistant biotypes with respect to kinetic parameters, to determine the nature of the interaction of inhibitors, and to assess the intricacies of their binding sites.

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