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Triclopyr Effects on Peroxidase Activity in Target and Non-target Aquatic Plants

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ABSTRACT

Rapid and characteristic physiological responses to herbicidal activity in aquatic plants have potential for monitoring treatment effect in laboratory evaluations and operational applications, or indicating contaminant runoff in aquatic ecosystems. Change in peroxidase enzyme (PRX) has been considered for use as such a parameter, and was investigated following application of the auxin-like herbicide triclopyr to four species at 1 mg ae/l for 12 hr, and 2.5 mg ae/l for 24 hr. Guaiacol-specific PRX increased rapidly within 1.5 days after triclopyr application in the dicot Eurasian watermilfoil (*Myriophyllum spicatum* L.). The non-target monocots elodea (*Elodea canadensis* Rich.), sago pondweed (*Potamogeton pectinatus* L.), and vallisneria (*Vallisneria americana* Michx.) showed no visual effects of triclopyr treatment through 8 DAT, and PRX levels were unchanged in treated and untreated plants during this time. However, by 35 DAT the 2.5 mg/l triclopyr rate (the maximum label rate) had reduced biomass of sago pondweed by 60%. The early PRX response to triclopyr effect which differentiated Eurasian watermilfoil from non-target species suggests that this parameter may be predictive of rapid susceptibility to this herbicide.

Key words: *Myriophyllum spicatum*, *Elodea canadensis*, *Potamogeton pectinatus*, *Vallisneria americana*, herbicide.

INTRODUCTION

The triethylamine salt formulation of the herbicide triclopyr ([3,5,6-trichloro-2-pyridinyl)oxy]acetic acid), Garlon^{R} 3A (DowElanco), is currently registered under a Federal experimental use permit (EUP) for control of nuisance aquatic plants. This compound has potential as a tool for restoration of habitat in the many North American aquatic ecosystems invaded by the exotic weed Eurasian watermilfoil (*Myriophyllum spicatum* L., hereafter referred to as milfoil) because of its ability to control this dicot species selectively (Getsinger and Westerdahl 1984, Netherland and Getsinger 1992). Triclopyr's auxin-type herbicidal activity generally controls woody and broadleaf species while most grasses and other monocots are tolerant (WSSA 1989). In aquatic ecosystems this differential response gives triclopyr the ability to remove milfoil and allow non-invasive native monocots and tolerant dicots to proliferate and provide wildlife habitat, sediment stabilization, and nutrient cycling (Getsinger et al. 1993).

Laboratory evaluations of concentration and exposure times required for triclopyr efficacy on milfoil have been validated by field studies (Netherland and Getsinger 1992, Getsinger et al. 1993), but the effects of triclopyr on non-target aquatic plants are less well-known. Certain species have been observed to recover and increase following field treatments that provide milfoil control (Getsinger et al. 1993, 1994). Information on the early physiological responses and long-term effects of triclopyr on a wide range of native aquatic vegetation will optimize the selective use of this herbicide.

Physiological and diagnostic parameters are being evaluated for the ability to provide information on herbicide response in aquatic plants and to reveal the mechanisms that confer tolerance (Sprecher and Netherland 1995). In the field this type of evaluation may serve to quantify short-term stress on desirable plants in a treated community and verify escape from permanent injury, or to detect herbicide movement to off-target populations following chemical application. Specific physiological changes related to the mode of action of a compound provide diagnostic tests for the presence of that compound, and may allow dose-related quantification of effect; however, metabolic responses to general physiological stress can also be useful as indicators of herbicide effect. One such response is the activation or increased synthesis of the oxidative enzymes (peroxidase, superoxide dismutase, catalase, glutathione reductase, polyphenol oxidase) which often occur in response to various biotic and abiotic stresses in plants (Lagrimini and Rothstein 1987, Graham and Graham 1991, Cakmak and Marschner 1992, Scandalios 1993). Certain herbicides induce rapid or long term modifications in the activity levels of these antioxidants, either directly due to mode of action or as an indirect result of general metabolic stress (Amsellem et al. 1993, Scandalios 1993).

Among aquatic macrophyte species, tolerance of waterborne pulp and paper mill effluent has been related to constitutively higher levels of peroxidase activity (PRX), and was attributed to the role of this enzyme in oxidizing xenobiotics (Roy et al. 1992). Investigation of PRX response in hydrilla (*Hydrilla verticillata* Royle) showed that levels of salt-extractable PRX increased with concentration of Cu^{2+}, or with increasing sub-lethal levels of a sulfonylurea herbicide (Byl and Klaine 1991, Byl 1992, Byl et al. 1994). Initial laboratory tests showed an increase in PRX enzyme activity in milfoil and hydrilla following fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]-4(1*H*)-pyridinone) treatment (Spre-

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cher et al. 1993b). Endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) treatment of hydrilla resulted in increased PRX, while levels in tolerant egeria (*Egeria densa* Planch.) remained unchanged (Sprecher et al. 1993a). Protein synthesis is also expected to increase in response to auxin herbicides, accompanying symptomatic growth regulator effects (Devine et al. 1993). Therefore, to investigate possible physiological parameters indicative of susceptibility, this study monitored PRX and total protein response following triclopyr treatment of three non-target monocot species versus the target dicot milfoil.

METHODS AND MATERIALS

The monocot species elodea (*Elodea canadensis* Rich.), sago pondweed (*Potamogeton pectinatus* L.), and vallisneria (*Vallisneria americana* Michx.), and the dicot milfoil were established in June 1993 from apical tips (elodea from ponds at the Lewisville Aquatic Ecosystems Research Facility, Lewisville, TX; milfoil from Suwanee Laboratories, Inc. Lake City, FL) or from tubers or winterbuds (sago pondweed and vallisneria from Wildlife Nurseries, Inc. Oshkosh, WI). Each species was randomly assigned to nine of 36 55-L aquaria, 28 × 28 × 72.5 cm, in a controlled-environment chamber. Ten 300-ml glass beakers filled with nutrient-enriched (Osmocote^R, 14:14:14) sediment from Brown's Lake, Vicksburg MS were planted with four apical tips or six underground propagules and placed in each aquarium. Aquaria were maintained under 14L:10D photoperiods of 541 ± 75 μE/m^2/sec of photosynthetically-active radiation (PAR) at a water temperature of 23 ± 2C. Simulated hard water (Smart and Barko 1984) was used, and except for the herbicide exposure periods an approximately half-volume water exchange was carried out in each aquarium three times a week, using a flow-through system. General conditions of establishment and growing environment were those previously described by Netherland et al. (1991) and Netherland and Getsinger (1992).

Pretreatment biomass was estimated from one randomly chosen beaker harvested from each aquarium 1 to 3 days before treatment. Roots were washed free of sediment and dried along with harvested shoots at 76C to a constant weight, and dry weights recorded. Treatment of individual species was initiated at weekly intervals to allow physiological analyses to be done on the same day samples were harvested. Stock solutions of triclopyr were prepared from Garlon^R 3A and added to each aquarium to achieve required concentrations. Triclopyr was applied at 0, 1.0 mg active ingredient (ae)/l for 12 hr, or 2.5 mg ae/l for 24 hr, to milfoil, sago pondweed, vallisneria, and elodea at 5, 6, 7, and 8 weeks, respectively, after planting. Treatments were arranged in a completely randomized design with three replications. Following exposure periods under static water conditions, aquaria were drained and refilled twice to remove triclopyr residues. Three untreated reference aquaria of each species were drained and refilled twice at the same time as aquaria treated for 24 hr. At 35 or 37 days after treatment (DAT) all beakers remaining in each aquarium were harvested and final biomass was determined in the same way as at pretreatment sampling.

After pretreatment biomass samples were taken, one beaker of each of the non-target species was placed in a milfoil aquarium, so that each of the latter contained six beakers of milfoil and one each of elodea, sago pondweed and vallisneria. These plants were visually evaluated during the course of the study for any effect from decomposition products of treated milfoil.

Peroxidase and total protein analyses. PRX levels and total protein content were measured 1 to 3 days before treatment and at 1.5, 8.5 and ≈35 DAT for all species, and in milfoil at 3.5 DAT. For both analyses, three 0.5 g fresh weight (g fw) portions of either shoot ≥5 cm distal to the apical tip (milfoil, elodea, sago pondweed) or fully-expanded leaf blade (vallisneria) were sampled from each aquarium. Milfoil and sago pondweed samples were macerated using a porcelain mortar and pestle while elodea and vallisneria samples were ground in a homogenizer (PRO Scientific Inc., Monroe, CT). Tissue was homogenized in 5 ml (sago pondweed, 10 ml) chilled 0.1 M Na_2PO_4 buffer, pH 6.1, to produce a crude extract which was centrifuged at 2,000 g for 5 min. The supernatant was filtered through one layer of Miracloth (Calbiochem Corp., San Diego, CA). Homogenization was carried out with chilled solutions on ice, and extracts were held at 4 C.

PRX was analyzed by combining 50 to 200 μL aliquots of the sample extract with 2.95 to 2.8 ml of a reaction solution to give a constant total reaction volume of 3 ml. The reaction solution contained 4 mM of the substrate guaiacol in 0.1 M Na_2PO_4 buffer, pH 6.1, with 3 mM H_2O_2 (Wang et al. 1991). Analyses were carried out in disposable polystyrene cuvettes. The reaction was initiated by combining sample extract with reaction solution and was monitored spectrophotometrically at 470 nm (Shimadzu UV/VIS 1201) for three minutes. PRX activity was calculated as change in absorbance (Abs) per minute after the first minute following reaction initiation, and reported as this unit rate per specified g fw of tissue sampled. Each extract was analyzed twice to give an average activity, and activities of the three subsamples taken from each aquarium were pooled to give mean activity of the treatment replication.

Total protein was quantified in the crude plant extract using preparations (BioRad Laboratories, Inc., Hercules, CA) based on the Bradford method (1976). A 100 μl aliquot of extract was reacted with 5 ml of Coomassie Brilliant Blue dye solution and read spectrophotometrically at 595 nm. Total protein in the sample was calculated from a standard curve of bovine serum albumin (BSA) protein.

Chlorophyll concentrations were determined from dimethyl sulfoxide extractions of three shoot samples from each aquarium of sago pondweed at 31 DAT using the method of Hiscox and Israelstam (1979).

Analyses of variance were performed on replicate means for PRX, protein and biomass data within each species for each sampling date. The Bayesian LSD test was used to separate means at the 95% confidence level (STAT-PACKETS 1.0, Minneapolis MN). Means are presented with standard errors except where noted.

RESULTS AND DISCUSSION

Biomass and physical effects. Pretreatment per-beaker biomass was 1.45 ± 0.15, 2.08 ± 0.36, 2.16 ± 0.38, and 1.72 ± 0.24 g dry weight (g dw) for milfoil, elodea, vallisneria, and sago pondweed, respectively. The estimate of standing milfoil bio-

mass calculated from 10 beakers per aquarium, 185 g dw $(m^2)^{-1}$, is similar to the average of seasonal maxima noted by Grace and Wetzel (1978) from field sites in the southeast.

Following treatment, only milfoil exhibited the epinastic curvature of apical and axillary shoots characteristic of auxin-like compounds. Symptoms occurred by 3 DAT and epidermal rupture was evident from presence of extracellular gas bubbles in stems. Treated plants became water-logged and began to decompose, and by 14 DAT no viable stems or leaves remained. No regrowth occurred in either treatment level, and no tissue remained for biomass harvest at 37 DAT (Table 1). Previous evaluations of triclopyr efficacy on milfoil indicated that treatment at 1.0 mg/l would not prevent regrowth after 2 weeks (Netherland and Getsinger 1992). This eradication may have been due to life cycle stage (phenology) at planting or treatment times, or to removal of apical shoots for analyses.

There were no significant differences in biomass among treated and untreated elodea and vallisneria 5 weeks after treatment (Table 1). There was a significant reduction ($p < 0.05$) in biomass of sago pondweed exposed to 2.5 mg/l triclopyr for 24 hr (Table 1). Auxin-related symptoms were not observed during the first week after herbicide application but by 20 DAT plants treated at both rates were exhibiting loss of turgor. Cellular deterioration continued in plants treated at the higher rate, indicated by tissue necrosis, colonization of the plant surface by algae, and paler-colored foliage. While chlorophyll content at 31 DAT did not differ significantly among treatments (1.11 ± 0.12, 1.12 ± 0.21, and 0.60 ± 0.28 mg (g^{-1}) with increasing concentration, $p = 0.22$), these plants had less than half the biomass of the other treatments at final harvest (Table 1).

These results confirm the potential of triclopyr to remove the exotic dicot milfoil while maintaining the native monocots elodea and vallisneria. Results are consistent with a field study in Washington State which found that elodea was among several native plants which increased substantially in the first and second year following removal of milfoil with applications of triclopyr (Getsinger et al. 1993, 1994). The loss of physical condition and reduced biomass in sago pondweed indicate that this species can be negatively affected by triclopyr. This is consistent with the observations of other researchers (L. W. J. Anderson, pers. comm.). However, the viable stems and root crowns that remained at 35 DAT at the maximum labelled rate could be expected to regenerate. Such a recovery has been observed in a field study where a decline in *Potamogeton* spp. at 28 DAT following triclopyr application was followed by 5- to 10-fold increases in biomass at one and two years posttreatment, with sustained milfoil eradication (Getsinger et al. 1993, 1994). Lower rates of triclopyr known to control milfoil (Netherland and Getsinger 1992) could be used where maintenance of sago pondweed is desirable.

There was no visible effect of decaying milfoil on the growth of the other species, and no evidence that *M. spicatum* releases allelopathic chemicals during decomposition following herbicide treatment.

Total protein. Protein content varied among species (Table 2). Protein content in vallisneria increased over the course of the study but was not affected by triclopyr treatment. In elodea, protein was significantly lower in treated shoots at 1.5 DAT but this difference was not observed at later sampling dates. Sago pondweed was not initially affected at 1.5 or 8.5 DAT but treated plants contained less than two-thirds of the protein found in untreated plants at 35 DAT.

Milfoil samples did not register a positive protein concentration value when read against the BSA standard curve (approx. 20 µg to 140 µg protein) in treated or untreated material at any of the sampling dates. Consequently, PRX was not reported relative to total protein content, although this is a frequently-used normalization for enzyme activity (eg. Roy et al. 1992). In subsequent experiments, analyses of milfoil using the Lowry method (Lowry et al. 1951; BioRad) produced measurable protein from 10 mg fw samples extracted in Na_2PO_4 buffer (data not shown). Thus, lack of response with the Bradford test was attributed to interactions between

TABLE 1. THE EFFECT OF TRICLOPYR CONCENTRATION AND EXPOSURE TIME ON THE DRY WEIGHT OF *E. CANADENSIS*, *P. PECTINATUS*, *V. AMERICANA*, AND *M. SPICATUM* 5 WEEKS AFTER TREATMENT.

Species	Triclopyr Treatment[1]	Biomass (g dry wt.)
Elodea canadensis	Untreated	26.34 ± 3.42[2]
	1 mg/1, 12 hr	25.33 ± 6.39
	2.5 mg/1, 24 hr	33.95 ± 5.37
Myriophyllum spicatum	Untreated	14.39 ± 0.69
	1 mg/1, 12 hr	0 ± 0.0
	2.5 mg/1, 24 hr	0 ± 0.0
Potamogeton pectinatus	Untreated	23.96 ± 1.29 a
	1 mg/1, 12 hr	20.02 ± 3.06 a
	2.5 mg/1, 24 hr	8.73 ± 0.74 b
Vallisneria americana	Untreated	22.04 ± 5.43
	1 mg/1, 12 hr	30.69 ± 3.58
	2.5 mg/1, 24 hr	23.81 ± 5.43

[1]Treatment means followed by different letters are significantly different as measured by a t-test, $p \leq 0.05$.
[2]Means followed by standard errors.

TABLE 2. THE EFFECT OF TRICLOPYR CONCENTRATION AND EXPOSURE TIME ON THE PROTEIN CONCENTRATION OF *E. CANADENSIS*, *P. PECTINATUS* AND *V. AMERICANA* TISSUE.

Species/Triclopyr treatment	Pre	1.5 DAT	8.5 DAT	35 DAT
	------- Total protein concentration, mg $(g^{-1}$ fw) -------			
Elodea canadensis				
Untreated	2.12 ± 0.14[1]	4.00 ± 0.28a[2]	5.19 ± 0.14	1.01 ± 0.20
1 mg/1, 12 hr		2.14 ± 0.29b	4.67 ± 0.98	2.27 ± 0.21
2.5 mg/1, 24 hr		2.72 ± 0.11b	3.80 ± 1.17	2.05 ± 0.45
Potamogeton pectinatus				
Untreated	4.92 ± 0.24	4.86 ± 0.24	4.47 ± 0.21	5.83 ± 0.54a
1 mg/1, 12 hr		3.04 ± 0.27	3.72 ± 0.48	3.40 ± 0.50b
2.5 mg/1, 24 hr		3.00 ± 0.36	4.04 ± 0.33	3.50 ± 0.14b
Vallisneria americana				
Untreated	1.53 ± 0.47	1.95 ± 0.13	2.72 ± 0.22	4.92 ± 0.42
1 mg/1, 12 hr		1.83 ± 0.18	3.11 ± 0.21	4.12 ± 0.36
2.5 mg/1, 24 hr		2.48 ± 0.33	3.04 ± 0.85	3.74 ± 0.46

[1]Means followed by the standard errors.
[2]Treatment means followed by different letters are significantly different as measured by a t-test, $p \leq 0.05$, within sampling date and species.

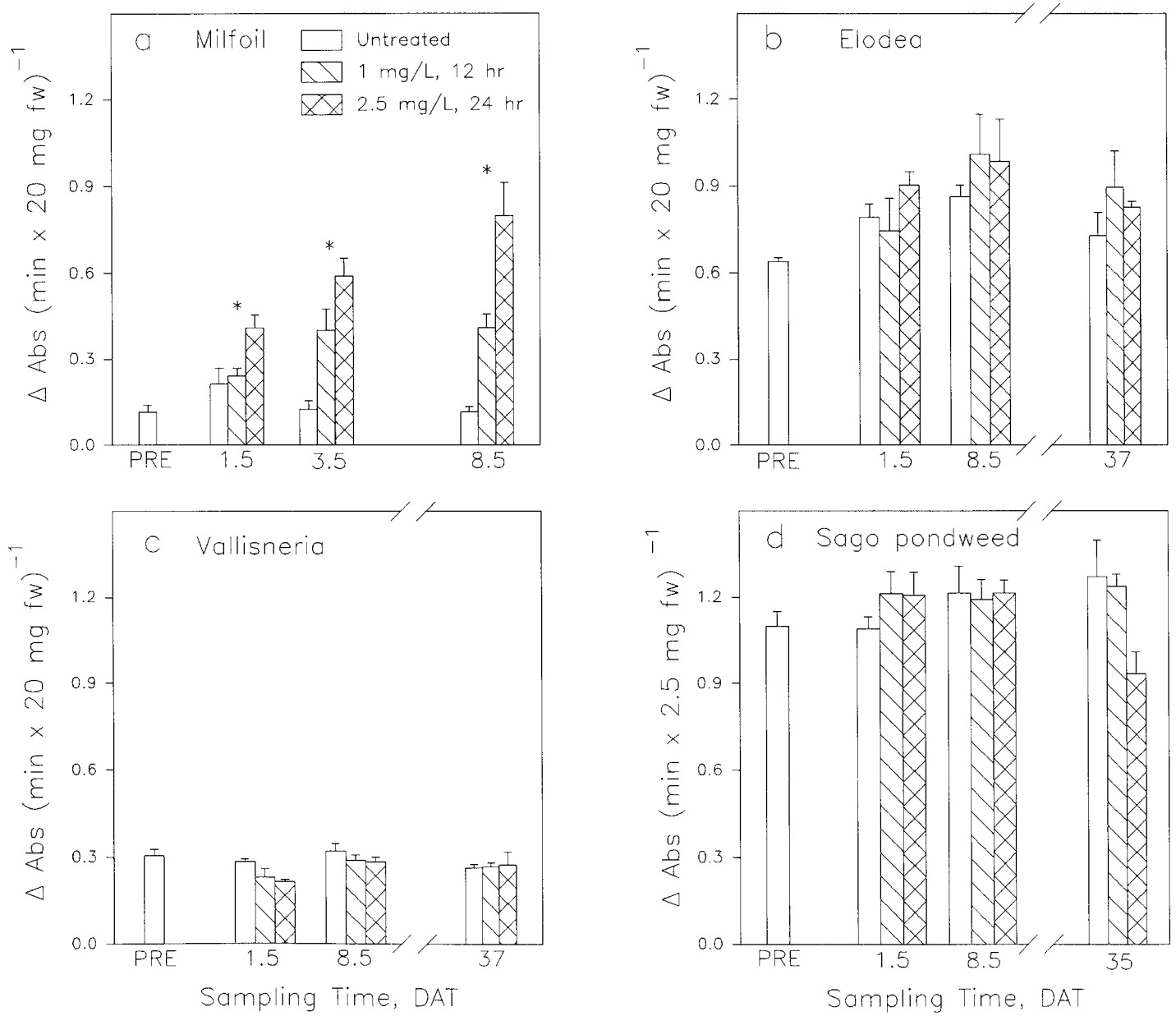

Figure 1. PRX activity in four species of aquatic plants treated with triclopyr at 0, 1.0 mg ae/l for 12 hr, or 2.5 mg ae/l for 24 hr. Measurements were taken at pretreatment, 1.5, 3.5, 8.5 and 35 or 37 days after treatment, and activity measured by a change in absorbance at 470 nm. Bars represent standard errors of means of three replicates. The presence of significant differences among treatments on the same sampling date (Bayesian LSD with $p \leq 0.05$) is noted with an asterisk.

compounds present in milfoil (eg. phenolics) and components of the test solution (Coomassie Brilliant Blue dye, phosphoric acid, methanol).

Peroxidase response. PRX activity in triclopyr-treated milfoil increased significantly over the sampling period. Untreated references did not vary from pretreatment levels during this period (overall mean activity = .152 ± 0.02, p = 0.36) (Figure 1a). At 1.5 DAT plants exposed to 2.5 mg/l for 24 hr had almost twice as much PRX activity as those in reference aquaria or those exposed to 1 mg/l for 12 hr. By 3.5 DAT, PRX in plants at the lower rate had increased significantly above untreated material and were similar to the 2.5 mg/l for 24 hr treatment. At 8.5 DAT, levels in the lower treatment remained the same, while activity in the higher treatment had increased to approximately seven times that of reference material. After this date loss of tissue integrity in treated plants precluded further PRX analysis.

Triclopyr treatment did not significantly alter PRX activity of elodea and vallisneria (Figure 1b and 1c). Although no increase was seen in sago pondweed at 1.5 and 8.5 DAT, at final harvest enzyme activity in plants treated with 2.5 mg/l for 24 hr had declined to about 75% of the combined mean of the reference and lower rate treatments (NS, p = 0.07, Figure 1d).

The rapid increase in PRX activity following triclopyr application was seen only in the susceptible milfoil, occurring by 1.5 DAT. This is consistent with the differential PRX response to endothall between hydrilla and egeria (Sprecher et al. 1993a). These findings indicate that increased enzyme activity results from generalized physiological stress not directly linked to herbicide mode of action, and suggest that increase in PRX activity in aquatic species can predict herbicide susceptibility. The decrease in PRX observed in sago pondweed treated at the higher dose is assumed to result from general loss of physiological competency, and to be qualitatively different from the rapid PRX increases seen here in milfoil with exposure to triclopyr. This parallels the absence of visible herbicide effect in the first week after treatment in this monocot. Variation from symptoms normally associated with auxin-like herbicide treatment in dicots may reflect difference in physiological response to triclopyr.

Constitutive levels of PRX varied among the species examined. Pretreatment PRX activities standardized to 20 mg fw tissue indicate constitutive levels of 0.116, 0.305, 0.639, and 8.78, in milfoil, vallisneria, elodea, and sago pondweed, respectively (Figure 1). These relative activities are consistent with published data from Roy et al. (1992) on species in the same genera. While higher constitutive levels were seen in the monocots, oxidizing ability of PRX enzyme did not preclude susceptibility to triclopyr in sago pondweed.

Comparable tests of other aquatic dicot species are required to confirm PRX response to triclopyr efficacy, and to determine the value of this enzyme as a predictive tool for assessing tolerance or susceptibility. In terrestrial plants, ethylene evolution has been found to be correlated to dose of growth regulator herbicides (Hall et al. 1985, Little et al. 1990, Hall et al. 1993). RNA acids and oxygen consumption, along with total protein levels, are also expected to increase following treatment with auxin mimics (Devine et al. 1993). Since increase in PRX is seen with ethylene production (Biles and Abeles 1991) and as a function of its activity as an indole-3-acetic acid oxidase (Quesada et al. 1992), it is possible that these other physiological responses occur with triclopyr exposure. As an indicator of relative herbicide effect or species susceptibility, PRX is easily measured and appears able to give early evidence of physiological response.

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