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Comparative Response of Two Hydrilla Strains to Fluridone

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ABSTRACT

Experiments were conducted in a controlled-environmental growth chamber to evaluate the response of two strains of the invasive submersed plant *Hydrilla verticillata* (L.f.) Royle to fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl)phenyl]-4(1*H*)-pyridinone). To assess plant injury, shoots were potted and placed in 10-L aquaria, grown to pre-canopy condition, then dosed with 0, 0.5, 5, 50, 500, and 5000 µg L^{-1} active ingredient (ai) fluridone for a 91-d exposure period. Apical tissues were analyzed for β-carotene pigment concentrations at intervals during the herbicide exposure period. The I_{50}, based on β-carotene concentrations, was 17.9 µg ai L^{-1} for Strain B and 3.68 µg ai L^{-1} for Strain A after 7 days. After 30 days, the I_{50} for Strain B was 47.8 µg ai L^{-1} and 3.14 µg ai L^{-1} for Strain A. For a plant biomass study, hydrilla shoots from Strains A and B were potted and placed in 52-L aquaria, grown to pre-canopy condition, then dosed with 0, 0.05, 0.5, 5, 50, 500, and 5000 µg ai L^{-1} fluridone for a 90-d exposure time. The GR_{50} for shoot biomass was 37.6 µg ai L^{-1} for Strain B and 5.78 µg ai L^{-1} for Strain A. Root biomass was negatively affected by fluridone concentrations, but not by strain. Based on these results, Strain B of hydrilla exhibited symptoms of fluridone resistance. With fluridone resistant hydrilla present in 20 Florida lakes, development of new chemistries with different modes-of-action is needed to establish a management program.

Key words: Hydrilla verticillata, submersed aquatic vegetation, bleaching herbicide, chemical control, β-carotene, herbicide resistance.

INTRODUCTION

The invasive submersed species, hydrilla (*Hydrilla verticillata* (L.f.) Royle) continues to cause serious problems in water bodies throughout the southern tier of the US. From

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its initial introduction near Tampa, Florida in the 1950s, the dioecious biotype of the plant has spread via vegetative reproduction to most watersheds within the state (Schmitz et al. 1991). In addition, DNA analysis has indicated that dioecious hydrilla colonies found growing in Florida, Texas, and California waters are likely from one common origin, close to accessions from Bangalore, India (Madeira et al. 1997, 1999). To check the spread of hydrilla and restore open water and native vegetation on large water bodies infested by the plant, low-dose applications ranging from 10 to 15 µg ai L^{-1} of the herbicide fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl) phenyl]-4(1*H*)-pyridinone) have been the management tool of choice (Netherland and Getsinger 1995a, 1995b, Fox et al. 1996). The widespread and frequent use of this herbicide is primarily due to the excellent cost-effective efficacy on hydrilla, and the low toxicological risk that fluridone poses to the non-target aquatic community and to human health.

Fluridone is considered a "bleaching herbicide," interrupting carotene biosynthesis in newly emerging tissue by blocking phytoene desaturase (PDS), an enzyme necessary for production of the intermediate pigment, phytofluene (Bartels and Watson 1978, Sandmann and Böger 1983). Because phytofluene is not produced, phytoene, another intermediate pigment, accumulates and the carotenoids α-carotene and β-carotene are not synthesized. Carotenoids are yellow pigments that aid in photosynthesis, and protect chlorophyll pigments from photoxidation under stressful photosynthetic conditions. Damaged chlorophyll limits the photosynthetic process, and plants eventually die. Visual symptoms of fluridone exposure are bleaching of actively growing plant apices as chlorophyll is destroyed via photooxidation in developing tissue (Bartels and Watson 1978). Plant apices may also appear light pink or purple when the duration or dose of fluridone increases as the anthocyanin pigments are unmasked and accumulate after chlorophyll photooxidation (Doong et al. 1993). Mature stems may remain green and continue to photosynthesize.

Resistance to PDS inhibitors, including fluridone, has been documented in both prokaryotic (Sandmann and Fraser 1993, Chamovitz et al. 1993, Windhövel et al. 1994) and eukaryotic (Vartak and Bhargava 1997) algae. Development of fluridone resistance was not thought likely or even possible for aquatic vascular plants, especially for hydrilla, since the dioecious (female) hydrilla biotype is known to reproduce by asexual means only. Nonetheless, the presence of fluridone-resistant hydrilla has been confirmed in Florida through a series of plant collections and analyses in water bodies throughout the state by Michel et al. (2004). Although hydrilla was found to be susceptible to fluridone in the majority of lakes, it was reported resistant in 20 lakes. The *in vivo* bioassays resulted in three levels of resistance to fluridone: low, intermediate, and high. Michel et al. (2004) determined that resistance occurred through mutations at the PDS gene and levels of resistance correlated to independent somatic mutations at this site. Moreover, because resistance factors at the enzymatic (*in vitro*) step matched responses by the plants in the *in vivo* assessments, these investigators suggested that the fluridone-resistant strains may be equally as competitive as the susceptible strains, and may persist as the dominant plant in a given water body even when selection pressure subsides after the dissipation of fluridone.

The present study was conducted in 2002 to compare the response of two hydrilla strains to fluridone, one of which was suspected of having tolerance or resistance to fluridone applications. Biomass production of both strains was compared under controlled-environmental conditions using a whole-plant dose response experiment because this type of evaluation is the preferred method for resistance verification (Beckie et al. 2000, Derr 2002). Comparisons were also made over time through evaluation of injury using plant shoot β-carotene concentrations. Use of β-carotene levels to document plant injury is specific to fluridone, revealing the onset of herbicide effect before visual symptoms occur (Sprecher and Netherland 1995).

MATERIALS AND METHODS

Plant Injury Study. Two strains of hydrilla were obtained from two different sources in Florida. Strain A was collected from an irrigation pond where hydrilla plants had never been exposed to fluridone. Strain B was collected from a lake that had a considerable history of fluridone treatments, and effective control of hydrilla was becoming increasingly difficult with fluridone herbicide.

Plants from each source were grown in 42 vertical aquaria (volume = 10 L) located in a walk-in controlled-environmental growth chamber (58 m^2). Twenty-one aquaria were used for each strain. Environmental conditions of the chamber were a light intensity of 520 ± 50 µmol m^{-2} sec^{-1}, temperature of 21 ± 2°C, and photoperiod of 14 h:10 h light:dark cycle. Lighting was provided by a combination of 400 watt high-pressure sodium and metal halide bulbs, fitted with glass plates to quench ultra-violet radiation that could degrade fluridone. Since the original concentration/exposure time relationships for fluridone against hydrilla were successfully developed in this experimental system (Netherland et al. 1993, Netherland and Getsinger 1995a, 1995b), it was determined that this system would be optimum for conducting a comparative study to document potential fluridone resistance.

Four healthy apical cuttings (15 cm) of hydrilla were planted in 300 ml glass beakers (diameter = 7 cm, depth = 12 cm) filled with natural lake sediment amended with 0.15 g L^{-1} ammonium chloride. A 1-cm layer of silica sand was added to the sediment surface to prevent suspension of sediment particles in the water column. Three beakers were placed in each aquarium, which was filled with a culture solution recommended for growth of submersed aquatic macrophytes (Smart and Barko 1984). Plants were allowed to establish for 21 days after planting.

A stock solution of an aqueous suspension fluridone formulation (AVAST!)[3] was prepared as 1 mg ai L^{-1}. A wide range of fluridone concentrations (0.5, 5, 50, 500, 5000 µg ai L^{-1}) were chosen following recommendations by Beckie et al. (2000) to document herbicide resistance in weeds. These concentrations were applied to the aquaria as plants were actively growing and starting to form a canopy. Untreated reference aquaria were

[3]Citation of trade names does not constitute endorsement or approval of the use of such commercial products.

included to assess plant physiology in the absence of herbicide exposure. Tissue samples (0.25 g) were collected from plant apices and analyzed for phytoene and β-carotene concentrations using the methods of Sprecher et al. (1998) at 0, 3, 7, 14, 30, 45, 59, and 91 days after treatment (DAT).

Each treatment, including the untreated reference, was replicated four times and blocked by strain. Two-way analysis of variance (ANOVA) was conducted to determine herbicide and strain effects on phytoene and β-carotene concentrations. Data were transformed using the square root to meet the assumptions of normality and equal variance. If there was a significant rate by strain interaction ($p \leq 0.05$), degree of plant resistance was determined through dose-response curves using herbicide concentration as the independent variable and untransformed pigment concentration data as the dependent variable. From the log-logistic dose response curve: $f = \min + (\max-\min)/(1+(x/I_{50})^{slope}$, the I_{50} was calculated using SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA) for both the resistant and susceptible strains (Seefeldt et al. 1995). The I_{50} indicates the herbicide dose that caused a 50% reduction in pigment concentrations.

Plant Biomass Study. Both strains A and B used in the previous plant injury experiment were grown in 28 vertical aquaria (volume = 48 L) located in controlled environmental growth chamber (58 m^2) with conditions described above.

Four healthy apical cuttings (15 cm) of hydrilla were planted in 300 ml glass beakers (diameter = 7 cm, depth = 12 cm) filled with natural lake sediment amended with 0.15 g L^{-1} ammonium chloride. A 1-cm layer of silica sand was added to the sediment surface to prevent suspension of sediment particles in the water column. Ten beakers, five for each strain, were placed in each aquarium, separated by plexiglass plates with holes that allowed for water circulation among the plants. Each aquarium was filled with a culture solution recommended for growth of submersed aquatic macrophytes (Smart and Barko 1984). Plants were actively growing (shoots ~50 to 70 cm in height) and starting to form a canopy (21 d) prior to herbicide application. Pretreatment biomass was estimated by removing plant shoots from two beakers, one beaker of each strain, in each aquarium. Shoots were dried at 70°C for 48 h then weighed to obtain a mean dry weight. Pretreatment shoot biomass (mean ± 1 SE) was 0.36 ± 0.02 g for susceptible plants and 0.36 ± 0.01 g for resistant plants. Each aquaria had shoot biomass similar to spring hydrilla biomass in Florida (Bowes et al. 1979).

A stock solution of an aqueous suspension fluridone formulation (AVAST!) was prepared as 5 mg ai L^{-1}. A wide-range of fluridone concentrations (0.05, 0.5, 5, 50, 500, 5000 µg ai L^{-1}) were chosen following recommendations by Beckie et al. (2000) to document herbicide resistance in weeds. These concentrations were applied to aquaria for a static exposure of 90 d. Untreated reference aquaria were included to assess plant growth in the absence of herbicide exposure. At 90 DAT, shoot and root biomass were harvested (4 beakers) from all treatments, dried at 70°C for 48 h, then weighed.

Each treatment, including the untreated reference, was replicated four times. Two-way ANOVA was conducted to determine herbicide and strain effects on shoot and root dry weights. If there was a significant rate by strain interaction ($p \leq 0.05$), degree of plant resistance was determined through dose-response curves using herbicide concentration as the independent variable and plant biomass as the dependent variables. Using the log-logistic dose response curve: $f = \min + (\max-\min)/(1+(x/GR_{50})^{slope}$ the GR_{50} was calculated (Seefeldt et al. 1995) using SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA) for both the resistant and susceptible strains (Seefeldt et al. 1995). The GR_{50} indicates the herbicide dose that causes a 50% reduction in plant growth.

RESULTS AND DISCUSSION

Plant Injury Study

Fluridone was present in the plant tissues as both hydrilla strains had depressed β-carotene levels as soon as 3 DAT for plants treated with fluridone concentrations ≥5 µg ai L^{-1} (Figure 1). Plants treated with 0.5 µg ai L^{-1} were similar to the untreated reference, supporting the results of Sprecher et al. (1998) who found that 2 µg ai L^{-1} was the minimum dose required to decrease β-carotene levels after 3 days. Plants treated ≥50 µg ai L^{-1} visually showed the characteristic bleached and purple stem apices at this time. Plants treated with 5 µg ai L^{-1} differed in response as fluridone inhibited β-carotene production in Strain A but not in Strain B at 3 DAT, and throughout the experiment.

Using β-carotene concentrations to compare plant injury (I_{50}), hydrilla Strain B was significantly more resistant than Strain A at 7 and 30 DAT. The I_{50} was 17.9 µg ai L^{-1} for Strain B and 3.68 µg ai L^{-1} for Strain A after 7 days, and 47.8 µg ai L^{-1} for Strain B and 3.14 µg ai L^{-1} for Strain A after 30 days (Table 1). The resistant/susceptible ratio (R/S) of 4.86 for the 7-day concentrations was similar to the R/S for a 14-day bioassay of a highly resistant hydrilla strain (Michel et al. 2004) while the R/S of 15.2 for the 30-day concentrations was substantially higher than published information. Changes in the R/S between 7 and 30 days reflect the difference in plant response in Strain B at doses between 5 and 50 µg ai L^{-1} (Figure 2). After 30 days, there were no significant differences between strains as both strains exhibited similar symptoms of injury.

Plant Biomass Study

Shoot weight reduction of both Strains A and B is shown in Figure 3. Shoot growth decreased as fluridone concentrations increased. Hydrilla Strain A had a GR_{50} of 5.78 µg ai L^{-1} while Strain B had a GR_{50} of 37.6 µg ai L^{-1} (Table 1). The GR_{50} for Strain A matches fluridone concentrations that have been proven lethal to hydrilla (5 to 10 µg ai L^{-1}) in concentration/exposure time small-scale studies (Netherland et al. 1993, Netherland and Getsinger 1995a, b) and in field trials (Haller et al. 1990, Fox et al. 1994, 1996). Alternatively, the GR_{50} of Strain B is higher than previous reports of 12 to 24 µg ai L^{-1} that suppressed shoot growth in small-scale studies using hydrilla collected from other Florida lakes (MacDonald et al. 2001), and according to Michel et al. (2004) indicates a high level of resistance.

The R/S for Strain B/Strain A of 6.5 for the plant biomass study is comparable to the 7-day bioassay R/S of 4.86 rather than the 30-day bioassay R/S of 15.2; however, the resistant and susceptible GR_{50} values required for a shoot reduction

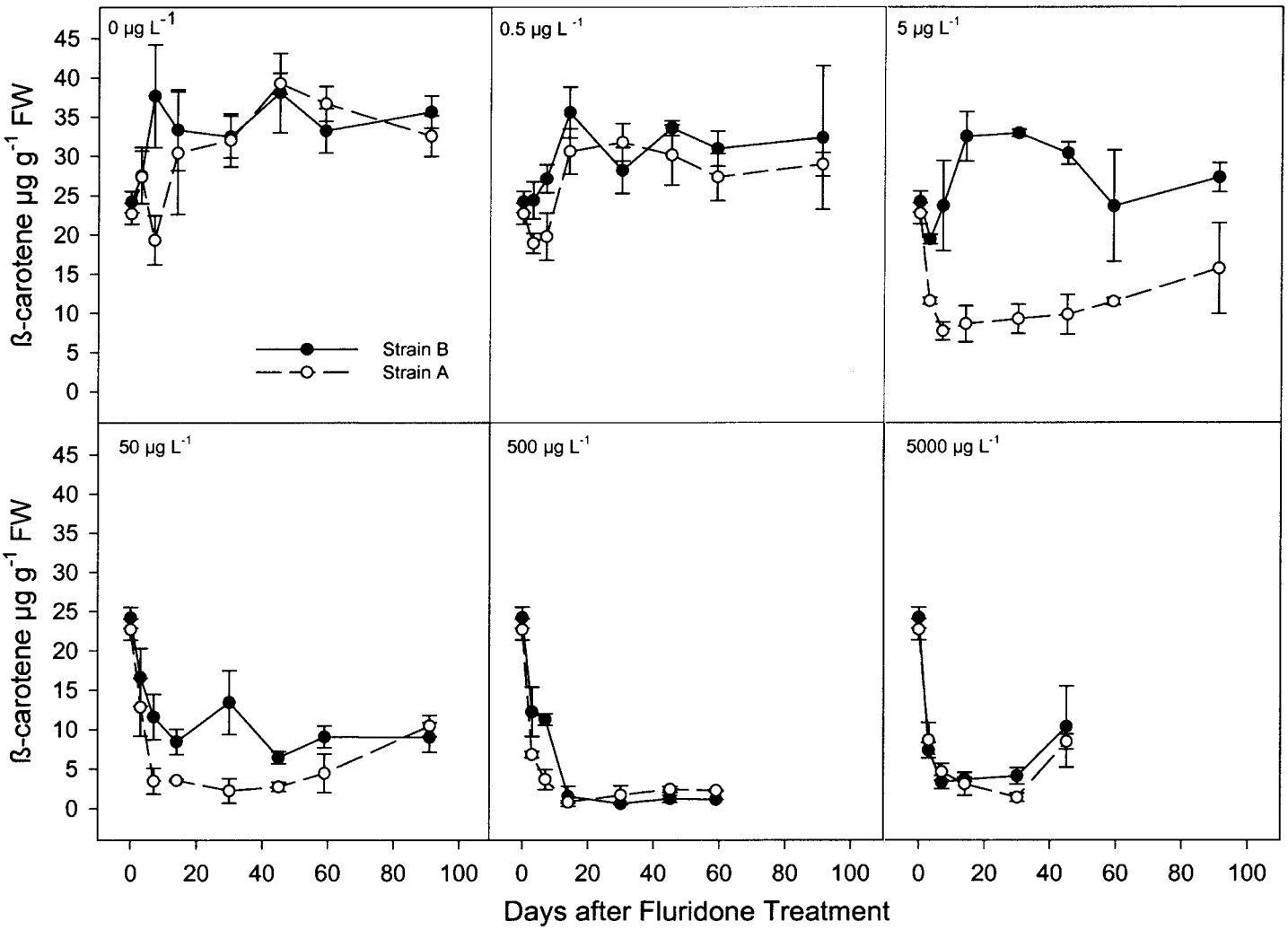

Figure 1. Concentrations of β-carotene (mean ±1 SE) in hydrilla strains A and B after exposure to 0, 0.5, 5, 50, 500, and 5000 µg ai L^{-1} fluridone. Plant tissues were sampled pretreatment (0) and 3, 7, 14, 30, 45, 59, and 91 days after treatment.

were 45 to 65% higher than the I_{50} values for plant injury (Table 1). While differences in these values may reflect the sensitivity of β-carotene concentrations to detect early changes in plant metabolism, plants may continue to grow and accumulate biomass despite fluridone exposure.

Plant root weight was significantly reduced by fluridone exposure ($F = 7.435$, $p < 0.001$; Figure 3), but not influenced by strain ($F = 1.076$, $p = 0.306$). Roots of both plant strains treated with rates of ≤5 µg ai L^{-1} were similar to the reference while root biomass of plants treated with higher rates was 50% less than the reference. With a root system intact, it is probable that over time regrowth will be greater in resistant hydrilla following fluridone treatment since there will be more shoot biomass to photosynthesize and translocate nutrients. These more robust plants will continue to thrive and reproduce thereby increasing resistant populations as described by Michel et al. (2004) and Arias et al. (2005).

According to the Weed Science Society of America (Heap 2005), "herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. Herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment." Given this definition, the results of these small-scale studies demonstrate that, when compared to hydrilla Strain A, the survival of hydrilla Strain B indicates that it is resistant to the herbicide fluridone. Doses of fluridone required to reduce shoot biomass in hydrilla Strain A in this study were comparable to those previously reported in

TABLE 1. GR_{50} AND I_{50} VALUES FOR TWO STRAINS OF HYDRILLA (A AND B) EXPOSED TO FLURIDONE (µG AI L^{-1}). THE GR_{50} VALUES WERE DETERMINED BY SHOOT DRY WEIGHT AFTER A 90-DAY EXPOSURE AND I_{50} WERE VALUES DETERMINED BY β-CAROTENE CONCENTRATIONS AFTER 7- AND 30-DAY EXPOSURES.

	7-day I_{50}	30-day I_{50}	GR_{50}
Strain B (Resistant)	17.90	47.80	37.60
Strain A (Susceptible)	3.68	3.14	5.78
R/S[1]	4.86	15.20	6.50

[1]Ratio of resistance to sensitivity calculated by dividing the GR_{50} or I_{50} of the resistant strain (B) by that of the susceptible strain (A).

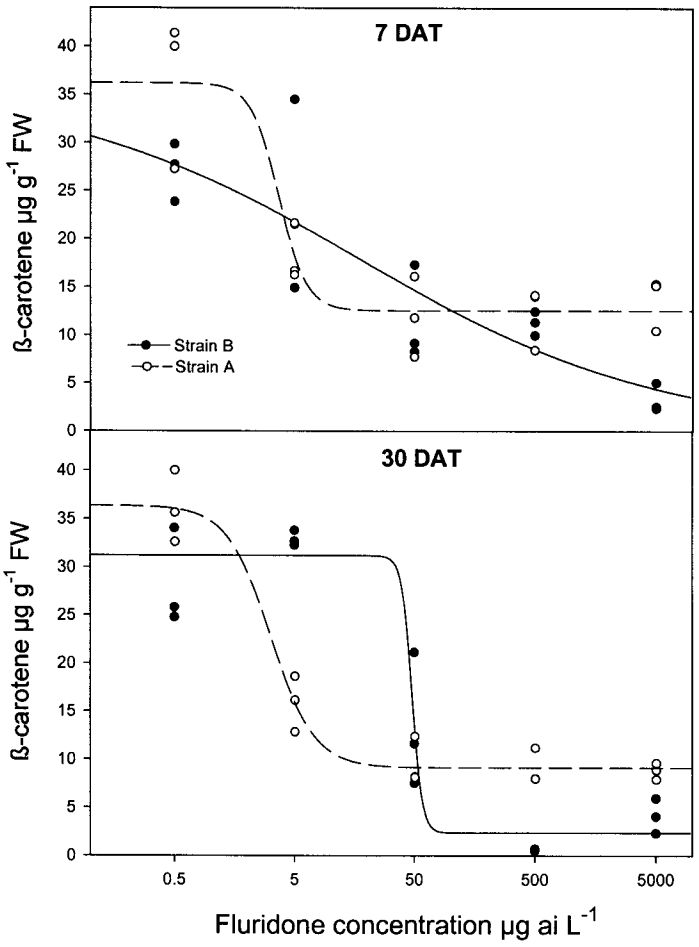

Figure 2. Concentrations of β-carotene in hydrilla strains A and B after a 7-day and 30-day exposure to fluridone. For 7-day exposure, dose-response curve for Strain B was $y = 0 + (35.7\text{-}0)/[1 + (x/17.9)^{0.35}]$, $R^2 = 0.76$, $p < 0.0001$ and dose-response curve for Strain A was $y = 3.92 + (19.8\text{-}3.92)/[1 + (x/3.68)^{3.78}]$, $R^2 = 0.85$, $p < 0.0001$. For 30-day exposure, dose-response curve for Strain B was $y = 2.36 + (31.2\text{-}2.36)/[1 + (x/47.8)^{10.4}]$, $R^2 = 0.93$, $p < 0.0001$ and dose-response curve for Strain A was $y = 1.76 + (32.1\text{-}1.76)/[1 + (x/3.14)^{2.39}]$, $R^2 = 0.96$, $p < 0.0001$.

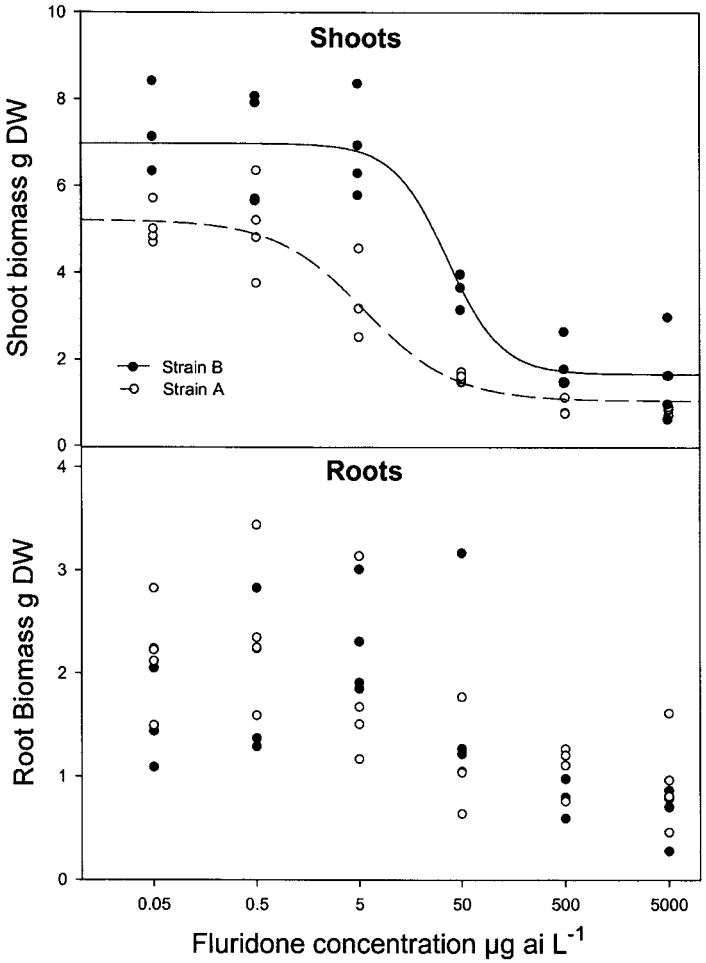

Figure 3. Shoot and root dry weight (DW) of hydrilla strains A and B after a 90-day exposure to fluridone. Dose-response curve for shoots of Strain B was $y = 1.63 + (6.95\text{-}1.63)/[1 + (x/37.5)^{1.66}]$, $R^2 = 0.88$, $p < 0.0001$. Dose-response curve for shoots of Strain A was $y = 1.04 + (5.21\text{-}1.04)/[1 + (x/5.78)^{1.00}]$, $R^2 = 0.98$, $p < 0.0001$.

studies conducted in the same experimental system (Netherland et al. 1993, Netherland and Getsinger 1995a, 1995b), although different fluridone formulations were used.

Currently, the only vascular plants that have been found to be resistant to PDS inhibiting herbicides are hydrilla and wild radish (*Raphanus raphanistrum* L.). Wild radish, a major dicot weed in Australia, developed resistance to diflufenican (*N*-(2,4 difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxamide) after four applications of this herbicide (Cheam et al. 2000). In plant biomass dose-response studies, 10 to 16% of plants survived four times the commercial application rate of diflufenican (Walsh et al. 2004). These studies also documented multiple-herbicide resistance in wild radish with herbicides across four modes-of-action, including a PDS inhibitor (diflufenican), an auxin analog, and two photosystem II-inhibitors.

This confirmation of multiple resistance in a terrestrial plant may have implications for how herbicides are used in aquatic systems. Reliance on a limited number of herbicides will likely result in weed resistance over time, as has been the case with sulfonylurea herbicides in rice (Kuk et al. 2003), acetyl-CoA carboxylase inhibitors in wheat (Beckie and Kirkland 2003), and acetolactate synthase (ALS) inhibitors in soybeans (Manley et al. 1998). Plant resistance to herbicides, although a new concept to aquatic plant managers, has been an issue in terrestrial weed control for decades. To date, there are 296 unique plant biotypes resistant to 18 classes of herbicides, with hydrilla as the only aquatic plant listed (Heap 2005). As resistant management strategies are implemented in lakes and reservoirs across the US, development of new chemistries with different modes-of-action is imperative for maintaining a suite of tools for controlling invasive aquatic weeds.

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