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Effects of Four Rice Herbicides on Seed Germination and Seedling Growth of a Threatened Vascular Plant *Penthorum chinense* Pursh

X.-Y. Luo, H. Ikeda

National Institute for Agro-Environmental Science, Kannondai 3-1-3, Tsukuba, 305-8604, Japan

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Penthorum chinense Pursh is a rooted vascular plant distributed in eastern Asia. A few decades ago, this plant was frequently observed in muddy wetlands, riparian flood plains, and fallow paddy fields in Japan (Kimura et al. 1999). However, its population has been decreasing in Japan and is now considered as a 'vulnerable' species by the Red Data Book of Japanese vascular plants (Environment Agency of Japan 2000). It is possible that rice herbicides are having adverse effects on non-target plants that grow around paddy fields such as *P. chinense*. However, for detecting the adverse effects of herbicides on non-target aquatic plants, micro-organisms (Peterson et al. 1994; Fairchild et al. 1997; Nyström et al. 1999; Junghans et al. 2003) and duckweeds (Peterson et al. 1994; Fairchild et al. 1997) have been mostly used, and little is known about the effect of rice herbicides on non-target rooted macrophytes (Lewis 1995). Since there has been an increasing public interest in biodiversity conservation, the toxicity of herbicides to endangered plants should be investigated. The seedlings of *P. chinense* emerge from late April to early May in Japan (Yonemura et al. 2000; Ikeda and Itoh 2001), and this term coincides with the time of application of herbicides in paddy fields. Therefore, it is expected that germinating seeds and seedlings of this species are exposed to rice herbicides. The objectives of this study are (1) to evaluate the effects of major rice herbicides which is currently used in Japan with different modes of action on the seed germination and seedling growth of this species, and (2) to assess the ecological hazard of runoff of these herbicides from paddy fields.

MATERIALS AND METHODS

As test paddy herbicides, we used bensulfuron-methyl (methyl α-[(4,6-dimethoxy pyrimidin-2-ylcarbamoyl)sulfamoyl]-*o*-toluate), mefenacet (2-benzothiazol-2-yloxy-*N*-methylacetanilide), simetryn (N^2,N^4-diethyl-6-methylthio-1,3,5-triazine-2,4-diamine) and thiobencarb (*S*-4-chlorobenzyl diethyl(thiocarbamate)), because these herbicides have been commonly used in Japan and have completely different herbicidal mechanisms. These herbicides belong to four different chemical groups, sulfonylureas, oxyacetamides, triazines and thiocarbamates, and inhibit acetolactate synthase, cell division, photosystem II and lipid synthesis, respectively. All substances were obtained from Kanto Kagaku (Tokyo, Japan). These compounds were initially dissolved in a small volume of acetone and

Correspondence to: H. Ikeda

diluted to the required concentrations with deionized pure water. The final acetone concentration was 1% (v/v) and did not affect the seed germination and seedling growth of *P. chinense*. The control was herbicide free but included 1% acetone. The herbicide solution was diluted with pure water for seed germination experiments and with a nutrient solution (25.69 mg L^{-1} $(NH_4)_2SO_4$, 4.11 mg L^{-1} $NaNO_3$, 50.43 mg L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 16.05 mg L^{-1} KCl, 15.28 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 26.25 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 6.60 mg L^{-1} EDTA-Fe, 0.45 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.072 mg L^{-1} H_3BO_3, 0.023 mg L^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.020 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.055 mg L^{-1} $ZnSO_4 \cdot 7H_2O$) for seedling growth experiments.

Mature seeds of *P. chinense* were collected from a natural population in a fallow paddy field in Tsukuba City, Ibaraki, Japan (36°7′N, 140°7′E, 25 m a. s. l) in 2002, and stored at 4°C in a refrigerator. The seeds were soaked in pure water and stored in a refrigerator for at least one week before use. The seeds were sterilized with 2% sodium hypochlorite solution for 20 min and then washed with pure water using a suction filtration appliance. Two layers of filter paper were placed in a plastic petri dish (55 mm in diameter) and 40 seeds were placed on it. After infusing 2 mL of herbicide solution, the petri dishes were covered and sealed with two layers of Parafilm®. The concentrations of each herbicide used were as follows: for bensulfuron-metyl 0.000003, 0.00003, 0.0003, 0.003, 0.03 and 0.3 µ*M*; for mefenacet 0.006, 0.06, 0.6, 6 and 60 µ*M*; for simetryn 0.004, 0.04, 0.4, 4 and 40 µ*M* and for thiobencarb 0.005, 0.05, 0.5, 5 and 50 µ*M*. There were three replications of petri dishes at each concentration of each herbicide. Then, the petri dishes were transferred to a plant incubator (EYELA FLI-301N, Tokyo Rikakikai Co., Tokyo, Japan) for germination. The photoperiod and temperature in the incubator were automatically controlled with a daily cycle of 14 hr light (220 µmol m^{-2} sec^{-1}) at 25°C and 10 hr dark (0 µmol m^{-2} sec^{-1}) at 15°C. Radicle emergence was the criterion in determining germination. Germination was recorded 4, 10 and 20 days after the initiation of herbicide treatment, and radicle length was measured with a microscope 20 days after the initiation of treatment.

Approximately 700 seeds were planted in a plastic tray (310 mm×220 mm×87 mm) containing vermiculite that had been watered, and kept in a plant incubator for germination and seedling growth. The photoperiod and temperature were automatically controlled with a cycle of 14 hr light (87 µmol m^{-2} sec^{-1}) at 25°C and 10 hr dark at 15°C. To retain moisture in the vermiculite and provide light for germination and seedling growth, the tray was covered with one layer of Saran Wrap® during the growth period. The seedlings were watered every week with nutrient solution after the 1st-leaf stage. For bioassays, 6-well (35 mm in diameter, 20 mm in depth), flat bottom microplates were used and 13 mL of herbicide solution were added to each well. Three holes (3 mm in diameter) were bored with an electric drill on the cover of each well. Seedlings at the 3rd-leaf stage were carefully picked out from the tray with vermiculite and their roots were washed with pure water. The roots of three seedlings were immersed in each herbicide solution from a hole and the microplates were transferred to the plant incubator. The concentrations for bensulfuron-metyl are 0.003, 0.03, 0.3, 3 and 30 µ*M* and those for the other herbicides are the same as the seed germination experiment. There was a microplate for each herbicide and the six levels of herbicide concentration including control were systematically allotted to six wells of each microplate. The photoperiod in the incubator was the same as that for pretreatment but the light intensity was 220 μmol m^{-2} sec^{-1}. To determine growth recovery after herbicide treatment, the herbicide solutions were replaced with nutrient solution after exposure for 10 days, and the seedlings were grown for another 10 days. Total fresh weight of three seedlings was measured prior to herbicide treatment, and 0 and 10 days after treatment.

All statistical analyses were conducted with SPSS 11.0J (SPSS Japan Inc., Hiroo, Tokyo, Japan), and relationships were considered significant when $P < 0.05$. To estimate the lowest observed effect concentration (LOEC) of herbicides on seed germination, Dunnett's test was performed on the difference between control (0 μM herbicide) and herbicide treatments for arcsine-transformed germination rates within an identical incubation time. To elucidate LOEC of herbicides on radicle elongation and seedling growth, the same test was also performed on the difference between control and herbicide treatments for radicle length and relative growth rate (RGR) of total fresh weight of seedlings, respectively. RGR was calculated by the equation, $(\ln W_2 - \ln W_1)/t$, where W_1 and W_2 are initial and final fresh weights of three seedlings and t is a growth period (d). A square root transformation and rank transformation were used for radicle length and RGR of seedlings, respectively to ascertain the homogeneity of variance. Moreover, to estimate the effective concentration of herbicides resulting in 50% inhibition (EC_{50}), the SPSS Probit Analysis procedure was performed using a log-logistic model (Seefeldt et al. 1995). Here, we assumed that the lower and upper limits of the response were zero and the control response, respectively. In addition, we ignored hormesis (response to treatment that exceeded the control) because there was no significant increase over the control response. We also ignored negative growth and used zero as the response when RGR showed negative quantities.

RESULTS AND DISCUSSION

All herbicides did not reduce percent seed germination, and more than 90% germination was observed even 4 days after the initiation of treatment. For radicle elongation, however, all herbicides showed greater inhibitory effects with increasing concentration (Fig. 1). Among these, bensulfuron-methyl showed the largest inhibition at and above 0.03 μM. Simetryn inhibited radicle elongation at and above 0.04 μM, and germinating seeds were all dead 13 days after the initiation of treatment at and above 0.4 μM. For the other herbicides, however, this lethal effect was not observed. In contrast to bensulfuron-methyl and simetryn, mefenacet and thiobencarb showed lower inhibitory effects on germinating seeds with inhibition of radicle elongation at and above 0.06 and 0.05 μM, respectively.

For seedling growth at the 3-rd leaf stage, all herbicides showed greater inhibitory effects with increasing concentration (Fig. 2). In mefenacet and thiobencarb, LOEC for RGR during treatment was lower than that for RGR after treatment. In bensulfuron-methyl and simetryn, however, LOECs for RGR during and after treatment were observed at the same concentration. The LOEC of bensulfuron-methyl was lower than that of the other herbicide for both RGR

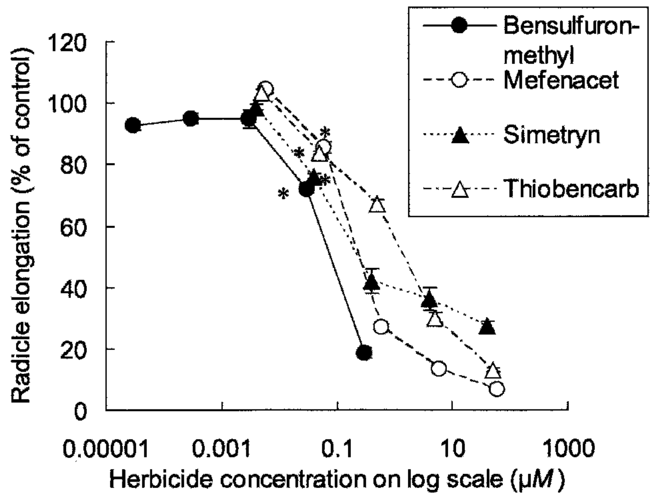

Figure 1. Effect of herbicides on radicle elongation of *Penthorum chinense* treated for 20 days. Vertical bars represent ±SE ($n = 3$). *: Lowest observed effect concentration.

during and after treatment, also indicating that the greatest inhibitory effect of bensulfuron-methyl on seedling growth during the 3-rd leaf stage.

For ecological risk assessment of pesticides, the Ministry of the Environment, Japan (2002) has proposed using EC_{50} as an endpoint for algae. Here, we obtained three measures for herbicide EC_{50}, radicle elongation during treatment, seedling growth during treatment and growth recovery after treatment (Table 1). For all herbicides, EC_{50} for seedling growth during treatment was lower, irrespective of the shorter exposure time, than that for radicle elongation of geminating seeds, indicating higher susceptibility to the herbicide during the 3rd-leaf stage. Moreover, except for simetryn, EC_{50} for growth recovery after treatment was higher than that for seedling growth during treatment, indicating that plant growth was recovered after treatment. Therefore, we adopted EC_{50} for growth recovery after treatment as the endpoint for hazard assessment: 3.5, 2600, 180 and 4500 n*M* for bensulfuron-methyl, mefenacet, simetryn and thiobencarb, respectively.

Among the herbicides we tested, EC_{50} for growth recovery after 10-d treatment were lowest for bensulfuron-methyl, indicating the most pronounced effect of bensulfuron-methyl on *P. chinense*. Bensulfuron-methyl is mainly used to control broadleaf and some sedge weeds in paddy fields (Japan Plant Protection Association 2001). Okamoto *et al.* (1998) reported that the duckweed *Lemna gibba* L. is more susceptible to bensulfuron-methyl than green algae, cyanobacteria and diatoms, and that the EC_{50} for the duckweed with exposure for 5 days (5-d EC_{50}) is 1.0 n*M*. Although the experimental conditions were different from each other, the 10-d EC_{50} for *P. chinense* growth was comparable to the 5-d EC_{50} for the duckweed, suggesting the susceptibility of *P. chinense* to bensulfuron-methyl. Okamoto *et al.* (1998) also investigated bensulfuron-methyl concentrations in drainage water from paddy fields on a nationwide scale in Japan

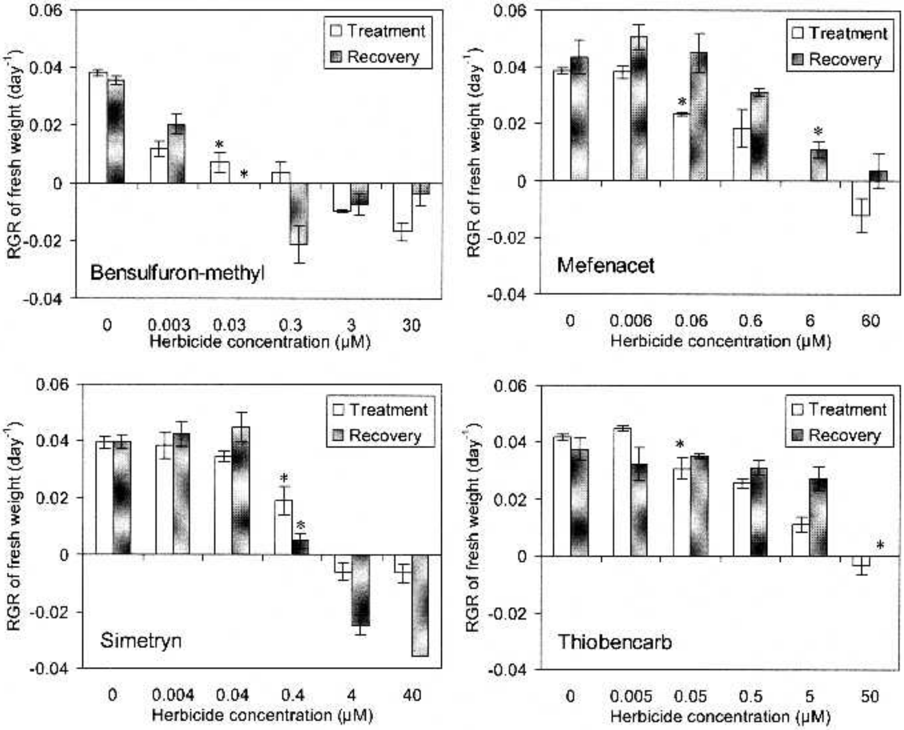

Figure 2. Effects of herbicides on relative growth rate (RGR) of total fresh weight of three *Penthorum chinense* seedlings at the 3-rd leaf stage during 10-d treatment (treatment) and during 10 days after treatment (recovery). Vertical bars represent ±SE (n = 3). *: Lowest observed effect concentration.

Table 1. Toxicity of herbicides for *Penthorum chinense*.

Herbicides	Germinating seed[a]	Seedling at the 3-rd leaf stage[a]	
	Radicle elongation during 20-d treatment	RGR[b] during 10-d treatment	RGR[b] recovery for 10 days after treatment
Bensulfuron-methyl	70 (23 - 390)	0.71 (0.00 - 3.8)	3.5 (2.7 - 4.4)
Mefenacet	400 (230 - 690)	210 (87 - 500)	2600 (1300 - 5100)
Simetryn	1300 (520 - 3900)	250 (100 - 620)	180 (140 - 220)
Thiobencarb	1400 (900 - 2100)	670 (330 - 1400)	4500 (500 - 310000)

[a] EC_{50} (nM) with 95% confidence intervals in parentheses,

[b] Relative growth rate of total fresh weight of three seedlings

from 1987 to 1993, and revealed that the highest concentration in 8 of 11 rivers in major rice-cropping areas is ranged from 0.24 to 5.6 nM and the longest duration bensulfuron-methyl detected ($\geq$ 0.24 nM) is ranged from 2 weeks to 1 month. The highest concentration in 3 of 11 rivers of Japan exceeds the EC_{50} for *P. chinense* growth recovery, and the maximum of the highest concentration is 1.6 times the EC_{50}.

Mefenacet is mainly used to control the grass weeds including *Echinochloa* spp. in paddy fields (Japan Plant Protection Association 2001). The highest mefenacet concentration in river water that has been reported in recent years (Nishimoto 1998; Okamura et al. 1999, 2002) is ranged from 21 to 88 n*M*, and these values are all lower than the EC_{50} for *P. chinense* growth recovery.

Only simetryn had lethal effects on the germinating seeds, but its 20-d EC_{50} for radicle elongation was 7 times higher than the 10-d EC_{50} for growth recovery after treatment during the 3rd-leaf stage. This herbicide is mainly used to control broadleaf and grass weeds in paddy fields (Japan Plant Protection Association 2001). It was previously found by Okamura et al. (2002) that the 3-d EC_{50} of simetryn for green algae growth is 56 n*M*, which is 1/3 the 10-d EC_{50} for *P. chinense* growth recovery. Also this herbicide is the most toxic component in the runoff to green algae among herbicides detected in river water around Lake Biwa, Japan. Hatakeyama et al. (1992) conducted bioassays on river water samples using the same green algae and found that most algal growth inhibition occurred during the end of May to late June was attributed to simetryn. Simetryn had the highest water solubility among the herbicides tested in the present study. Because there is a positive correlation between herbicide water solubility and their runoff from paddy fields (Maru 1991), high levels of simetryn runoff after application are expected. However, recent studies (Ministry of the Environment, Japan 2002; Okamura et al. 2002; Sudo et al. 2002) showed that the highest concentration of simetryn detected in river water is ranged from 30 to 108 n*M*, and these concentrations do not exceed the EC_{50} for *P. chinense* growth recovery.

Thiobencarb is also mainly used to control the grass weeds including *Echinochloa* spp. in paddy fields because of its high selectivity to rice (Japan Plant Protection Association 2001). It was determined by Okamura et al. (2002) that the 3-d EC_{50} of this herbicide for the green algae is 140 n*M*, which is lower than any of the EC_{50} values for *P. chinense*. The highest concentration of thiobencarb in river water that has been reported in recent years (Nishimoto 1998; Okamura et al. 2002; Sudo et al. 2002) is ranged from 6.8 to 24 n*M*, and all of these values are lower than the EC_{50} for *P. chinense* growth recovery.

In the present study, we conducted exposure experiments with constant herbicide concentrations under controlled light and temperature conditions. However, the real fields in which *P. chinense* is distributed are different from those experimental conditions. Firstly, most rice herbicides are applied once in paddy fields in Japan and herbicide runoff usually shows a transient pulse of concentrations over time. Therefore, the plants which have been exposed to herbicide runoff are able to grow after the herbicide concentration decreases to the level of no effects. In fact, *P. chinense* seedling growth recovered to some extent after being transferred from low-middle levels to herbicide-free conditions except for simetryn. It is suggested that exposure experiments including growth recovery after treatment is useful to evaluate the ecological impact of rice herbicide runoff.

Secondly, we estimated herbicide toxicities for the emergent plant *P. chinense* using a hydroponic culture in the present study, in which only roots were exposed to herbicides, while we used maximum herbicide concentrations in the surface water of rivers to assess the ecological impact of herbicide runoff. Herbicide concentrations in the river water could differ from those in the soil water around roots of the test species, making our assessment uncertain. Most herbicides used here are adsorbed mainly on the surface soil in paddy fields (Inao and Kitamura 1999; Japan Plant Protection Association 2001), and thus roots of juvenile seedlings soon after germination are likely to be exposed to higher herbicide concentrations than those in the surface water, suggesting that the toxicity of herbicide runoff depends greatly on the plant growth stage. Considering that herbicide concentrations in the rhizosphere water of rivers in Japan have not been reported, measurements of those concentrations would make the ecological hazard assessment of herbicides more certain for plants rooted in the soil.

The results presented here show that bensulfuron-methyl is most toxic to the threatened plant *P. chinense* among the four rice herbicides used, and suggest that bensulfuron-methyl concentrations that have been previously detected in the river water of some rice-cropping areas in Japan reach levels likely to cause adverse effects on seedling establishment of this species. Aida et al. (2004) conducted an exposure experiment using pots with a paddy soil under natural light and temperature conditions, and demonstrated that the bensulfuron-methyl runoff levels in Japan are also expected to have adverse effects on the growth of threatened aquatic ferns, *Azola japonica* Franch. et Savat., *Marsilea quadrifolia* L. and *Salvinia natans* All. Therefore, susceptibilities to sulfonylurea herbicides for threatened vascular plants should be taken into account to reduce the ecological risk from herbicide runoff in aquatic environments.

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