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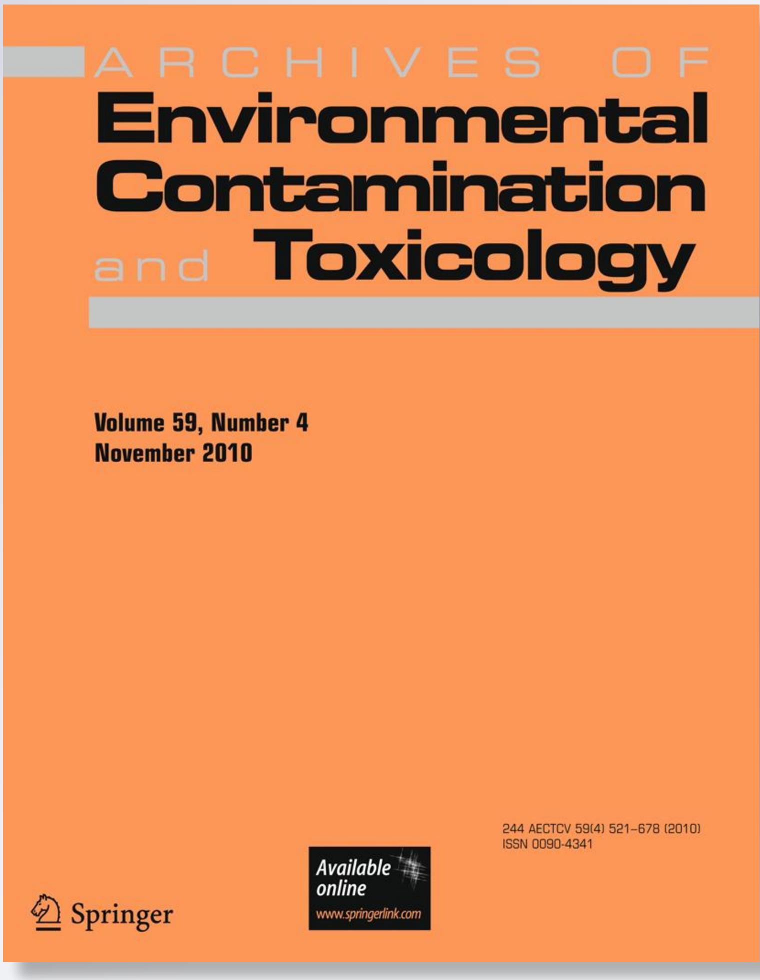
Archives of Environmental Contamination and Toxicology

ISSN 0090-4341
Volume 59
Number 4

Arch Environ Contam Toxicol
(2010) 59:574-581
DOI 10.1007/
s00244-010-9519-0



Springer

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DOI 10.1007/s00244-010-9519-0

Effect of Three Insecticides and Two Herbicides on Rice (*Oryza sativa*) Seedling Germination and Growth

M. T. Moore · R. Kröger

Received: 20 January 2010 / Accepted: 29 March 2010 / Published online: 13 April 2010

Abstract Rice (*Oryza sativa* L.) is one of the most important food crops worldwide. However, it is also a valuable tool in assessing toxicity of organic and inorganic compounds. For more than 20 years, it has been an approved species for standardized phytotoxicity experiments. The objective of this study is to determine germination and radicle (root) and coleoptile (shoot) growth of rice seeds exposed to three insecticides and two herbicides, commonly used in the agricultural production landscape. Although no germination effects of pesticide exposure were observed, significant growth effects were noted between pesticide treatments. Coleoptile growth was significantly ($p \leq 0.05$) lowered in metolachlor/atrazine mixture, diazinon, and lambda-cyhalothrin exposures when compared with controls. Radicles of fipronil-exposed seeds were significantly larger ($p \leq 0.05$) when compared with controls. This research contributes to the phytotoxicity assessment database, in addition to laying the foundation for the use of rice as a phytoremediation tool for agricultural pesticide runoff.

The first gathering and cultivation of rice (*Oryza sativa* L.) dates from between 12,000 and 6,000 years ago, providing a rich history for undoubtedly one of the most important cereal crops in the world (Huggan 1995). Second only to wheat (*Triticum aestivum* L.) in world production, nearly 600 million tons of rice are produced annually on about 150 million ha (Delseny et al. 2001). According to Ricestat (2008) an average of 1,061,000 ha of rough rice have been planted annually from 1960 to 2007 in the USA alone. Today, rice is a staple for nearly 3 billion people, providing 50–80% of their daily caloric intake (Delseny et al. 2001). The global importance of rice as a food source is evident; however, it is also a valuable tool in assessing potential phytotoxicity of organic and inorganic compounds. Phytotoxicity is defined as the impact or damage that a compound causes on certain plant characteristics (e.g., growth rate, germination, or root and shoot development). Rice has been recommended by the Organisation for Economic Co-operation and Development (OECD) for use in standardized phytotoxicity assessments for over two decades (OECD 1984). Additionally, several studies have demonstrated rice's increased sensitivity to complex effluents (Wang 1990, 1991; Wang and Keturi 1990) as compared with other commonly used seed species (e.g., lettuce). Phytotoxicity assessments are generally underutilized in ecotoxicology studies when compared with animal (e.g., fish, macroinvertebrate) assessments (Wang and Williams 1988). Root and shoot development occurs during seed germination, and studies have indicated increased sensitivity in the root system, as opposed to the shoot, when evaluating effects of contaminants (Wong and Bradshaw 1982). Very few studies have examined the phytotoxicity of commonly used pesticides on rice germination and growth (root and shoot development) in the USA. Therefore, the goal of this research is to examine five commonly used pesticides (atrazine, metolachlor, diazinon, fipronil, and lambda-cyhalothrin) for their effects on germination and root and shoot development of rice.

M. T. Moore (✉)
Water Quality and Ecology Research Unit, USDA-ARS National Sedimentation Laboratory, Oxford, MS 38655, USA
e-mail: matt.moore@ars.usda.gov

R. Kröger
Department of Wildlife and Fisheries, Mississippi State University, PO Box 9690, Mississippi State, MS 39762-9690, USA

The triazine herbicide atrazine (2-chloro-4-ethylamine-6-isopropylamino-*S*-triazine) is one of the most commonly used and scientifically studied herbicides in the USA. Registered for use in 1959, it continues to be applied to corn, sorghum, and sugar cane for protection against weeds under trade names such as Aatrex™ and Atrazine 4L™. Likewise, it is the most commonly used corn herbicide in combination with conservation and no-till land practices, which are vital land practices in the battle against soil erosion (Henderson 2007). An estimated 34.8 million kg of atrazine was used annually in US agriculture applications between 1999 and 2004 (USGS 2009). Atrazine is also used in combination with other herbicides for successful weed control. One such product is Bicep™, a formulation containing atrazine and the chloroacetanilide herbicide *S*-metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-((1*S*)-2-methoxy-1-methylethyl)acetamide]. While not as intensively used as atrazine, metolachlor estimated annual agricultural use between 1999 and 2004 was still over 11.1 million kg (USGS 2009).

Diazinon [*O*,*O*-diethyl-*O*-2-isopropyl-6-methyl (pyrimidine-4-yl)phosphorothioate] is an organophosphate insecticide sold only for nonresidential or agricultural applications, with nearly 280,000 kg of active ingredient being applied annually between 1999 and 2004 (USGS 2009). Fipronil [5-amino-1[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-(trifluoromethylsulfinyl)-1*H*-pyrazole-3-carbonitrile] is a new-generation phenyl-pyrazole insecticide registered in 1996. While individual uses for fipronil widely vary, it is a broad-spectrum insecticide used intensively in corn and rice production. USGS (2009) estimates that over 190,000 kg of fipronil was used annually in US agricultural applications between 1999 and 2004, with 94% being applied to corn crops. Lambda-cyhalothrin [(*RS*)-alpha-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate] is a fourth-generation pyrethroid insecticide used in soybeans, cotton, corn, and rice. Over 106,000 kg of lambda-cyhalothrin was applied each year to agricultural fields between 1999 and 2004 (USGS 2009). Intensive use along the Mississippi Delta corridor (Arkansas, Tennessee, Mississippi, and Louisiana) has raised concerns about the pesticide's potential impact on water quality.

Materials and Methods

Pesticides

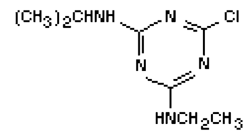
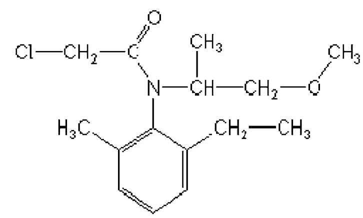
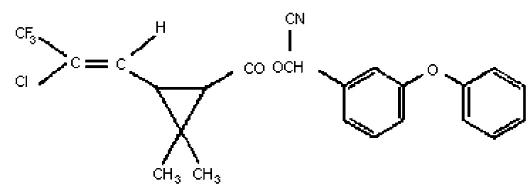
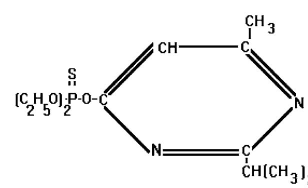
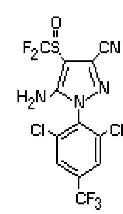
Germination and growth effects of *O. sativa* seeds (Drew® variety; certified 80% germination) exposed to Karate™ (lambda-cyhalorhtin), Diazinon 4E™ (diazinon), Regent™ (fipronil), Aatrex 4L™ (atrazine), and Bicep™ (atrazine + metolachlor) were examined. Chemical structures, and physical and chemical properties of the respective pesticides can be found in Tables 1 and 2. Commercial formulations of the insecticides and herbicides were used in both single exposures and mixtures. Because both expected environmental concentrations and effects data vary by orders of magnitude for pesticides used in the current study, the targeted individual pesticide concentration used in this study was 10 μg L^{-1}. Although this concentration is slightly lower than the US Environmental Protection Agency (USEPA) acute vascular plant aquatic life benchmarks for atrazine (37 μg/l) and metolachlor (21 μg/l), it still provided sufficient analytical sensitivity for experimentation (USEPA 2009). Initial aqueous samples of pesticides and pesticide mixtures were collected prior to seed introduction for analysis.

Seed Germination, and Coleoptile and Radicle Growth

Germination experiment protocols followed Stevens et al. (2008). Each pesticide treatment consisted of seven replicates of 15 individual *O. sativa* seeds per replicate, for a total of 105 total seeds per treatment. Replicates consisted of 15 seeds placed in a 25 × 150 mm FisherBrand® borosilicate glass culture tube. Fifteen milliliters of each pesticide or pesticide mixture was added to corresponding treatments and replicates. Each control treatment replicate received 15 ml deionized (DI) water, which was analyzed to ensure no pesticide contamination. After addition of the pesticide (or DI water), culture tubes were capped with parafilm, maintained at room temperature (23°C), and inverted three times every 30 min for 2 h. After the 2-h period, rice seeds and remaining liquid in each replicate were transferred to a prelabeled 100 × 10 mm Kimax® Petri dish containing a single Whatman® no. 1, 90-mm filter paper. Lids were placed on the dishes and they were transferred to a plant growth chamber (model DT 70SDF; Powers Scientific Inc.). Temperature in the chamber was maintained at 30 ± 2°C with complete darkness for the 4-day exposure period.

At the end of 4 days, dishes were retrieved from the growth chamber and three variables were recorded on each seedling: germination, coleoptile length, and radicle length. According to Stevens et al. (2008) germination was deemed impaired if neither coleoptile nor radicle was ≥1 mm in length. Data were recorded for each replicate and then all germinated seeds for each treatment were consolidated into a 25 × 150 mm glass tube. Ten milliliters of a 90:1 mixture of hexane:acetone were added to each glass tube containing treatment seedlings. The solution was allowed to sit for 30 min, after which seeds were removed from tubes and placed on clean Kimax® Petri dishes for drying. The solution was then analyzed for pesticide residue attributable to wash-off. After drying to constant weight, seedlings were ground using a mortar and

Table 1 Chemical structures of atrazine, metolachlor, lambda-cyhalothrin, diazinon, and fipronil

Atrazine	
Metolachlor	
Lambda-cyhalothrin	
Diazinon	
Fipronil	

pestle and analyzed for the respective pesticides. Nonsignificant statistical differences still exhibiting biological increases or decreases were deemed stimulatory effects.

Pesticide Analyses

All pesticide analyses were conducted on a gas chromatograph (model 7890A; Agilent) equipped with dual autoinjectors (7683B series; Agilent), dual split-splitless inlets, dual capillary columns, and Agilent® ChemStation. Autoinjector was set at 1.0 µl injection volume fast mode for all targeted pesticide analyses (Smith and Cooper 2004; Smith et al. 2007). The gas chromatograph was equipped with two micro electron capture detectors (µECDs). Primary analytical columns used were Agilent® HP 5MS capillary columns, 30 m × 0.25 mm i.d. × 0.25 µm film thickness, with the exception of diazinon, which used a 1 MS capillary column. Column oven temperatures and retention times

Table 2 Physical and chemical properties of atrazine (A), metolachlor (M), lambda-cyhalothrin (LC), diazinon (D), and fipronil (F)

	A	M	LC	D	F
Molecular weight (g/mol)[a,b,c,d]	215.7	283.8	449.9	304.4	473.2
Water solubility (mg/L)[a,c,e]	33.0	530	0.005	40.0	1.90–2.4
Vapor pressure (mm Hg)[a,b,f]	3×10^{-7}	1×10^{-5}	1.5×10^{-9}	7.3×10^{-7}	2.8×10^{-9}
Log K_{OW}[a,d,g,h]	2.68	3.28	7.00	3.30	3.9–4.1
Log K_{OC}[a,g,i,j,k]	1.40–2.19	2.27	5.26	3.00–3.27	2.92
Hydrolysis ($t_{1/2}$) (days)[b,c,j,l,m]	244	>200	233	138	>100

[a] EXTOXNET (Extension Toxicology Network) (1996)
[b] USEPA (1980)
[c] Casjens (2002)
[d] Gunasekara and Troung (2007)
[e] Tomlin (2000)
[f] Rhône-Poulenc Ag Company (1998)
[g] USDA (1995)
[h] Schroer et al. (2004)
[i] Ciba-Geigy Corporation (1994)
[j] Kegley et al. (2007)
[k] Ying and Kookana (2001)
[l] Li and Feldbeck (1972)
[m] Bobe et al. (1998)

differed slightly between pesticides. Only the parent compound was analyzed for atrazine, metolachlor, diazinon, and lambda-cyhalothrin. In the case of fipronil, the metabolites fipronil desulfinyl, fipronil sulfide, and fipronil sulfone were also analyzed in addition to the parent compound. Limits of analytical detection in aqueous samples were as follows: fipronil sulfide = 0.02 μg L^{-1}; metolachlor = 0.1 μg L^{-1}; lambda-cyhalothrin, diazinon, fipronil, fipronil sulfone, and fipronil desulfinyl = 0.2 μg L^{-1}; atrazine = 2.0 μg L^{-1}. Limits of detection for seedling (plant) samples were as follows: fipronil sulfide = 2.0 μg kg^{-1}; metolachlor = 10 μg kg^{-1}; lambda-cyhalothrin, diazinon, fipronil, fipronil sulfone, and fipronil desulfinyl = 20 μg kg^{-1}; atrazine = 200 μg kg^{-1}.

Statistical Analyses

Radicle and coleoptile data set comprised a significant amount of zero, or no growth, data points. As these data were of interest, the data was non-normally distributed and violated assumptions of one-way analysis of variance (ANOVA). Significant differences in radicle and coleoptile growth lengths were compared between pesticide treatments and controls using Kruskal–Wallis ranked analysis of variance. Two post hoc tests were performed: (1) Tukey honestly significantly different (HSD) tests to highlight those treatments that were significantly different, and (2) Dunnett's method, where the control group was predesignated and significant differences between pesticide treatments and control evaluated pesticide effects. Alpha was set at 0.05.

Results

Exposure Verification

All aqueous stock solutions (including control DI water) were analyzed for measured target pesticide concentrations. Control water was free from contamination, while the atrazine stock measured 7.47 μg L^{-1} (10 μg/l initial target for all pesticide concentrations). Two Bicep™ stock exposures were created. The first Bicep™ stock, based on initial 10 μg L^{-1} atrazine target, measured 5.47 μg Lμg L^{-1} atrazine and 4.48 μg L^{-1} metolachlor. The second Bicep™ stock, based on initial 10 μg L^{-1} metolachlor target, measured 18.4 μg L^{-1} atrazine and 7.52 μg L^{-1} metolachlor. Measured diazinon stock solution was 6.43 μg L^{-1}, while lambda-cyhalothrin stock measured 5.22 μg L^{-1}. Fipronil stock solution had the following components: fipronil desulfinyl (below detection), fipronil sulfide (0.781 μg L^{-1}), fipronil sulfone (0.543 μg L^{-1}), and fipronil (13.0 μg L^{-1}). A stock solution containing an equal parts mixture of diazinon, lambda-cyhalothrin, and fipronil had the following concentrations: diazinon (2.93 μg L^{-1}), lambda-cyhalothrin (1.88 μg L^{-1}), fipronil desulfinyl (below detection), fipronil

sulfide (0.427 μg L^{-1}), fipronil sulfone (0.289 μg L^{-1}), and fipronil (5.44 μg L^{-1}).

Pesticide Effects

No statistically significant differences ($p \geq 0.05$) were noted in either radicle or coleoptile growth in seedlings exposed to atrazine (only) versus control seedlings (Table 3). A slight stimulatory effect was noted in radicle growth (15% increase compared with controls), while atrazine-exposed seedlings exhibited a 1% decrease in coleoptile growth. Analysis of seedling rinse indicated no detectable concentration of atrazine, while the ground, germinated seedlings had a concentration of 55.0 μg kg^{-1}.

As previously mentioned, two different exposures were prepared for Bicep™ (atrazine + metolachlor). One utilized a target concentration of 10 μg L^{-1} atrazine in the Bicep™ mixture, while the second had a targeted metolachlor concentration of 10 μg L^{-1}. Hereafter, they will be referred to as atrazine/metolachlor and metolachlor/atrazine mixes. In the atrazine/metolachlor mixture, there were no significant differences in radicle or coleoptile growth when compared with controls. Atrazine/metolachlor-exposed seedlings demonstrated a growth decrease of 6 and 9%, respectively, for radicles and coleoptiles when compared with controls (Table 3). The seedling rinse of the atrazine/metolachlor mixture yielded 2.32 μg L^{-1} atrazine, and metolachlor concentrations were below detection. However, when ground seedlings were analyzed, both atrazine and metolachlor concentrations were below detection. When considering the second mixture (metolachlor/atrazine), slightly different results were obtained. Although no significant difference was noted in radicle growth when compared with controls (1% decrease), coleoptile growth decreased by 21%, which was statistically significant from the control coleoptile growth ($\chi^2 = 16.07$; df = 8; $p = 0.0414$). Metolachlor concentrations were below detection in both the seedling rinse and ground seedlings; however, atrazine concentrations were 0.867 μg L^{-1} and 23.0 μg kg^{-1} for the rinse and seedlings, respectively.

Diazinon-exposed seedlings did not demonstrate statistically significant differences in either coleoptile or radicle growth when compared with controls. A 13% decrease in coleoptile growth and 15% increase in radicle growth, both stimulatory effects, were noted when compared with control results (Table 3). Concentrations of diazinon in seed rinse were below detection limits, while ground seeds contained 5.85 μg kg^{-1} diazinon.

A statistically significant ($\chi^2 = 4.626$; df = 4; $p > 0.3279$) stimulatory effect was observed in fipronil-exposed seedling radicles (97% growth increase) as compared with controls, although coleoptile growth was decreased by only 1% (Table 3). In the seed rinse, fipronil desulfinyl was below detection limits, while fipronil sulfide, fipronil, and fipronil sulfone were present at concentrations of 0.477, 5.34, and 2.42 μg L^{-1}, respectively. When the ground seeds were analyzed, only fipronil (4.14 μg kg^{-1}) and fipronil sulfone (0.470 μg kg^{-1}) were present in detectable concentrations.

Lambda-cyhalothrin-exposed seedlings showed no statistically significant difference in coleoptile growth, although a stimulatory effect (17% decrease) was noted when compared with controls. Once again, a stimulatory effect was observed in radicle growth (31% increase), although it was not statistically significant (Table 3). Seedling rinse analysis indicated concentrations of 3.25 μg L^{-1}, while seedling concentrations measured 4.51 μg kg^{-1}.

For the insecticide mixture (equal parts diazinon, fipronil, and lambda-cyhalothrin), no statistically significant differences were noted in either radicle or coleoptile growth when compared with controls. Although a stimulatory effect was noted in radicle growth (14% increase), coleoptile growth decreased by only 4%. The following insecticide measurements were observed in the seedling rinse: fipronil sulfide (0.485 μg L^{-1}), fipronil (3.50 μg L^{-1}), fipronil sulfone (1.96 μg L^{-1}), and lambda-cyhalothrin (1.84 μg L^{-1}). Fipronil desulfinyl and diazinon were both below detection limits in the seedling rinse. Only fipronil

Table 3 Seed germination and mean radicle and coleoptile growth of rice exposed to pesticides ($n = 105$ seeds per treatment) after 4 days treatment exposure

Treatment	Mean ± SE		
	Germination (%)	Radicle (mm)	Coleoptile (mm)
Control[a]	80 ± 10	3.93 ± 4.83	14.0 ± 7.88
Lambda-cyhalothrin	80 ± 7	5.14 ± 6.76	11.7 ± 6.76
Fipronil	76 ± 8	7.52 ± 5.52*	13.6 ± 6.88
Diazinon	85 ± 8	4.61 ± 5.69	12.1 ± 5.90
Mix[b]	81 ± 10	4.38 ± 5.10	13.4 ± 7.66
Control[c]	79 ± 7	4.01 ± 0.39	12.7 ± 0.51
Atrazine (only)	72 ± 12	4.62 ± 0.30	12.6 ± 0.53
Atrazine[d]/metolachlor	81 ± 10	3.78 ± 0.61	11.6 ± 0.87
Metolachlor[e]/atrazine	78 ± 8	3.95 ± 0.53	10.0 ± 0.47*

SE standard error

[a] Control performed alongside insecticide experiment

[b] Mixture contained lambda-cyhalothrin, fipronil, and diazinon, each at 10 μg L^{-1}

[c] Control performed alongside herbicide experiment

[d] Mixture of atrazine and metolachlor was based on 10 μg L^{-1} atrazine target

[e] Mixture of metolachlor and atrazine was based on 10 μg L^{-1} metolachlor target

*Statistical significance from control

(4.00 μg L^{-1}) and fipronil sulfone (0.770 μg L^{-1}) were measured in ground seedlings. All other insecticides, including diazinon and lambda-cyhalothrin, were below detection limits.

Overall, all pesticides and mixture exposures, with the exception of the metolachlor/atrazine mixture, resulted in increased rice radicle development when compared with controls. Increases ranged from 6% (atrazine/metolachlor mixture) to 97% (fipronil). With the exception of the atrazine/metolachlor mixture, all pesticide and mixture exposures led to decreased coleoptile development when compared with controls, ranging from 1% (atrazine) to 21% (metolachlor/atrazine mixture).

Discussion

Interest in plant uptake of organic contaminants dates back almost half a century to studies conducted by Lichtenstein (1960) and King et al. (1966). Selected studies conducted in the last two decades have advanced the science even further (Burken and Schnoor 1997; Trapp and Matthies 1995; Trapp 2004; Weiss 2000). While many of these studies examined plant uptake with regard to phytoremediation, there are also concerns with contamination of food crops by pesticides through either foliar or root uptake. Root uptake of contaminants through passive or active processes may result in translocation through the plant transpiration system to other components (Briggs et al. 1982; Chiou et al. 2001; Li et al. 2005; Su et al. 2005).

Rice seeds were used in the current study because the grain is consumed globally at the rate of 400 million metric tons, accounting for nearly half of total cereal consumption, with approximately half of the world's population reliant on it for sustenance (Zhu et al. 2008). Likewise, as reported by Su and Zhu (2006), few published studies exist on rice plant uptake of anthropogenic substances. Of the published studies, most deal with uptake of various metals by rice. Wang (1994) examined toxicity of eight metals to rice seed and found that copper induced the most seed germination toxicity, while manganese induced the least amount of toxicity. Bhattacharyya et al. (2005) reported that rice straw chromium levels were higher than levels found in rice grain. Cadmium and arsenic effects on rice have also been studied. Rice straw and roots contained significantly greater amounts of five metals, including cadmium and arsenic, than did rice grain (Liu et al. 2007). Rahman et al. (2007a) found that arsenic concentrations in roots were 28-fold higher than in shoots and 75-fold higher than in raw rice grain. In a separate study, Rahman et al. (2007b) reported that, as the arsenic concentration in soil increased, arsenic concentrations in different parts of all studied rice varieties also significantly increased. However, arsenic concentrations were still below the World Health Organization's permissible limit of 1 mg/kg for human consumption (Rahman et al. 2007a).

A handful of recent studies have examined pesticide effects on rice. Wang (1994) reported rice seed toxicity exposed to paraquat, 2,4-D, glyphosate, and bromacil. A comparison of herbicidal activity effects on germination and seedling growth of rice and hemp sesbania was conducted by Hirase and Molin (2002). Other studies have examined specific pesticide and other organic contaminant effects on rice (Su and Zhu 2007; TenBrook and Tjeerdema 2006). The current study showed that measureable concentrations had adsorbed to rice seeds during germination (coleoptile, radicle, and seed). Furthermore, analysis of pesticide concentrations sorbed within the seeds showed that all treatments, except metolachlor, had detectable concentrations.

Only two pesticides utilized in the current study (atrazine and fipronil) have been examined with regard to effects on rice germination or uptake. Su et al. (2005) reported on the uptake of atrazine by rice seedlings, determining that both atrazine and cadmium were toxic to seedlings. Later studies (Su and Zhu 2006, 2007) determined a decrease in rice seedling transpiration as atrazine concentrations increased, emphasizing the necessity to account for different transport pathways of organic compounds in plant roots. In the current study, no atrazine or atrazine mixture effects were noted on rice radicle (root) growth. The metolachlor/atrazine mixture in the current study resulted in significantly decreased coleoptile growth when compared with controls. This is not completely unexpected, since metolachlor's mode of action is as a seedling shoot growth inhibitor. It is important to keep in mind, however, that referenced studies continued to examine rice at later growth stages, whereas the current study examined only a 4-day exposure.

Stevens et al. (1999) examined fipronil's effects on rice germination and documented that 4-day exposure at 2,000 mg L^{-1} significantly impaired germination; however, this was clearly not a realistic field exposure concentration. According to the USGS (2003), maximum fipronil concentrations in water ranged from 0.83 to 5.3 μg/L during tailwater release from a rice field in March and April. In the current study, seeds exposed to fipronil at nominal concentrations of 10 μg L^{-1} showed no significant germination impacts, although there was a significant stimulatory effect on radicle growth of rice seeds. Both increased shoot length and root system dry weights were noted in field plots using fipronil as a rice seed treatment versus malathion (Stevens et al. 1998). Rice et al. (1996) reported significant increases in plant height from fipronil-treated plots, suggested possible stimulatory effects. The USEPA (1998) established permanent tolerances for fipronil residue on rice grain of

0.04 mg/kg, well below concentrations measured from rice grains in the current study.

As evidenced by the sparse literature on phytotoxicity of pesticides on rice, it is difficult to compare results of the current study with those from any previous research. Increased interest in using rice as a possible plant for phytoremediation in agricultural pesticide runoff suggests it is crucial that more research be conducted on seedling germination and growth effects. Rice is also subjected to organic pollutants in the natural growing cycle in countries such as China, which relies heavily on this grain for human consumption and sustenance. For this reason, the current study is a valuable resource for determining possible effects of organic contaminants on rice.

Acknowledgments Thanks to Lisa Brooks and Forrest Briggs for sample preparation. Appreciation is also extended to Renee Russell for pesticide analyses and T & J Farms for rice seed donation. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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