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Mammalian Cell Cytotoxicity Analysis of Soybean Rust Fungicides

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The identification of soybean rust, caused by *Phakopsora pachyrhizi* H. Sydow & Sydow, in the southern United States in November 2004 (Schneider et al. 2005), in the Midwest in 2006 (Hartman et al. 2007), and elsewhere has increased the concerns of the impact of the pathogen on US soybean production (Miles et al. 2007). The rapid spread of *P. pachyrhizi* and its potential to cause severe yield losses makes this among the most destructive foliar diseases of soybean. Yield losses of 20%–60% were reported in Asia, with losses of 80% reported from experimental plots in Taiwan (Hartman et al. 1991). Soybean rust could have a major impact on both total soybean production and production costs in the US.

The primary control of soybean rust has been with fungicides (Miles et al. 2003). There are four fungicides commercially available in the US that are registered for use on soybean and labeled for soybean rust. These fungicides are Bravo®, Echo®, Headline®, and Quadris®. Headline® and Quadris® are strobilurin products; Bravo® and Echo® are chlorothalonil products. In addition, there have recently been Section 18 Emergency Exemption requests for additional fungicidal compounds (myclobutanil, tebuconazole, propiconazole, tetraconazole, and trifloxystrobin) and mixtures of compounds (trifloxystrobin and propiconazole; propiconazole and azoxystrobin; and pyraclostrobin and tebuconazole) submitted to the EPA by the Departments of Agriculture from many states (U.S. EPA 2006a). A number of commercial products (e.g., Bumper®, UpperCut®, Quilt®, Laredo®, and Stratego®) containing these requested fungicidal compounds have been approved and may now be registered (Section 3 registration status) for management of soybean rust on soybean (U.S. EPA 2006a).

While these fungicides have been used on crops (e.g., fruits, vegetables, and cereals), many questions remain regarding the large-scale application of these compounds to soybeans (~74 million acres in the US) in the prevention and treatment of soybean rust. Indeed, there is growing concern about the use of these fungicides since little is known about the toxicity of many of these compounds and their impact on human health. Therefore, the goals of this project were to assess the toxicity of soybean rust fungicides (Table 1) using a mammalian cell cytotoxicity assay and to compare the cytotoxicity (%C1/2) of these fungicides to the published toxicity values of other agrichemicals (insecticides and herbicides).

Materials and Methods

General laboratory reagents and supplies were purchased from Fisher Scientific Co. (Itasca, IL) and from Sigma-Aldrich Co. (St. Louis, MO). Media supplies and fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT). The fungicides used in this study (Table 1) were analytical grade of myclobutanil, tebuconazole, propiconazole, and trifloxystrobin purchased from Sigma-Aldrich and tetraconazole from Crescent Chemical

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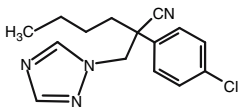
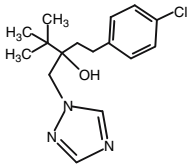
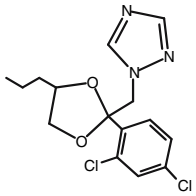
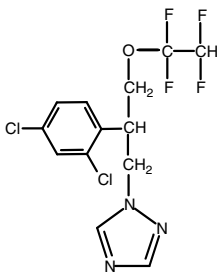
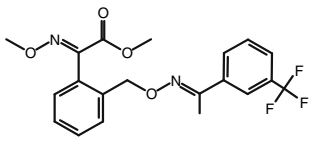
Table 1 Characteristics of fungicides[a]

Common name (H_2O solubility; mg/L at 20°C)	Chemical name (CAS number)	Chemical structure	Mode of action
Myclobutanil (142)	α-butyl-α-(4-chlorophenyl)-1H-1,2,4-triazole-1-propanenitrile(88671-89-0)		Inhibitor of sterol synthesis
Tebuconazole (36)	(RS)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (107534-96-3)		Inhibitor of sterol synthesis
Propiconazole (110)	1-((2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl)methyl)-1H-1,2,4-triazole(60207-90-1)		Inhibitor of sterol synthesis
Tetraconazole (159)	1-[2-(2,4-dichlorophenyl)-3-(1,1,2,2-tetrafluoroethoxy) propyl]-1H-1,2,4-triazole(112281-77-3)		Inhibitor of sterol synthesis
Trifloxystrobin (0.61)	benzeneacetic acid, (E,E)-α-(methoxyimino)-2-[[[[1-[3-(trifluoromethyl)phenyl]ethylidene]amino] oxy]methyl]-,methyl ester(141517-21-7)		Inhibitor of cellular respiration

[a] Sources of information (Dow Agrosciences 2005; U.S. EPA 2006b)

Co. (Islandia, NY). Concentrated (1 M) stock solutions of the fungicides were prepared in dimethyl sulfoxide (DMSO) and used in the mammalian cell cytotoxicity assay. Chinese hamster ovary (CHO) cell line AS52 (clone 11-4-8) was maintained in sterile complete F12 medium, consisting of Ham's F12 medium with 5% FBS, 1% antibiotics (100 units/mL sodium penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B), and 1% glutamine, at 37°C in a humidified atmosphere of 5% CO_2 (Wagner et al. 1998).

The chronic mammalian cell cytotoxicity assay was performed as previously described (Sorensen et al. 2003; Attene-Ramos et al. 2006). Flat-bottomed, tissue culture 96-well microplates were used; eight replicate wells were prepared for each fungicide concentration. Eight wells were reserved for the blank control consisting of 200 μL of the complete F12 medium. The negative control consisted of eight wells containing 100 μL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 μL of the complete F12 medium. The wells for the remaining columns contained 3,000 CHO cells, the complete F12 medium, and a known concentration of fungicide for a total volume of 200 μL. Fungicides, initially prepared in DMSO, were diluted in the complete F12 medium to achieve the desired final concentration; DMSO in the medium did not exceed a maximum final concentration of 0.5%. To prevent cross-over contamination between wells due to volatilization of the fungicidal agents, a sheet of sterile AlumnaSeal

(RPI Corp., Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The plate was placed on a rocking platform for 10 min to uniformly distribute the cells, and the microplate was placed in a tissue culture incubator. After 72 h of incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 30 min with a 1% crystal violet solution in 50% methanol. The plate was rinsed and 50 µL DMSO was added to each well. Plates were incubated for 30 min at room temperature and then analyzed at 595 nm with a Bio-Rad (Hercules, CA) microplate reader. The average absorbance of the blank control wells (no cells) was subtracted from the absorbance value of each test well. The mean value for the negative control (cells with complete F12 medium only, no fungicide) was set at 100%, and the absorbance value for each treatment group (i.e., fungicide concentration) well was converted into a percentage of the negative control. From regression analysis of the concentration–response data, a %C1/2 value (i.e., the fungicide concentration that reduced the CHO cell density by 50% as compared to the negative control) was determined for each fungicide. To determine if differences from the negative control were significant, data were subjected to a one-way analysis of variance.

Results and Discussion

The CHO microplate chronic cytotoxicity assay is a rapid, cost-effective method for the *in vitro* analysis of environmental compounds and has been recently used to quantitatively assess the toxicity of a variety of agrichemicals (Sorensen et al. 2003; Wagner et al. 2005), drinking water disinfection by-products (Plewa et al. 2002; Cemeli et al. 2006), and other toxic chemical agents (e.g., H_2S) (Attene-Ramos et al. 2006). In the present study, all of the soybean rust fungicides produced a significant reduction in CHO cell density after 72 h (Fig. 1). The observed reduction in CHO cell density was probably the result of fungicidal cytotoxicity; in this assay, the reduction in cell density might also be due to the fungicides inhibiting cell growth or disrupting the cell cycle. Thus, the fungicide concentration that caused a 50% reduction in the CHO cell density as compared to the negative control was referred to as the %C1/2 value rather than as the LC_{50}.

The %C1/2 values of the fungicides (calculated from regression-based analysis of data from concentration–response curves in Fig. 1) exhibited a 150-fold range from a value of 110.9 µM for myclobutanil to 0.7 µM for trifloxystrobin (Table 2). Based on %C1/2 values, the rank order of the fungicides relative to decreasing toxicity was trifloxystrobin > tetraconazole > propiconazole > tebuconazole > myclobutanil.

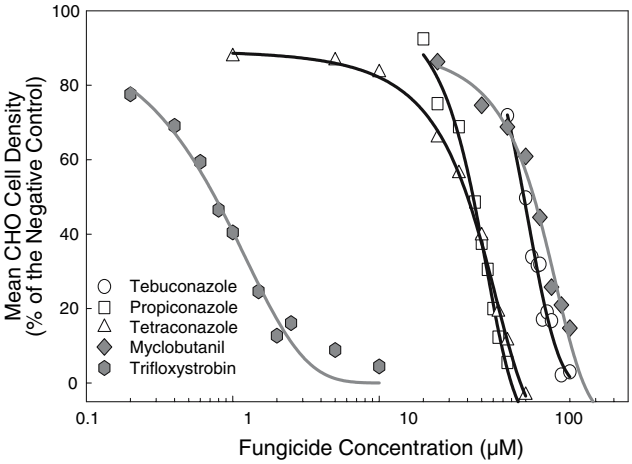


Fig. 1 Concentration–response CHO cell cytotoxicity curves for the fungicides tebuconazole, propiconazole, tetraconazole, myclobutanil, and trifloxystrobin. See Table 2 for r^2 value of the regression analysis for each curve

Table 2 Mammalian cell cytotoxicity of fungicides

Name	CR (µM)[a]	ANOVA test statistic[b]	r^{2c}	%C1/2 (µm)[d]
Myclobutanil	25–300	$F = 58.2$ ($p \leq 0.001$)	0.99	110.9
Tebuconazole	75–200	$F = 62.9$ ($p \leq 0.001$)	0.99	98.1
Propiconazole	20–90	$F = 41.6$ ($p \leq 0.001$)	0.99	44.0
Tetraconazole	1–100	$F = 59.5$ ($p \leq 0.001$)	0.99	39.2
Trifloxystrobin	0.2–10	$F = 117.6$ ($p \leq 0.001$)	0.99	0.7

[a] CR, selected concentration range

[b] Analysis of variance test of the data presented in Fig. 1

[c] The coefficient of determination for nonlinear regression equations that were used to calculate the %C1/2 value

[d] Calculated concentration of the fungicidal agent that reduced the CHO cell density to 50% of the negative control

All five of the soybean rust fungicides displayed a significant toxic response (Table 2). In fact, the %C1/2 values for these compounds were considerably lower than the reported %C1/2 value (4.25 mM) for ethyl-methanesulfonate (EMS), a well-studied toxicant and mutagenic agent (Wagner et al. 1998; Plewa et al. 2002). Among the fungicides tested, the triazoles (myclobutanil, tebuconazole, propiconazole, and tetraconazole) as a group were 50 to 150-fold less cytotoxic than the strobilurin (trifloxystrobin). What specific factors contributed to the differences in toxicity of these fungicides to CHO cells have yet to be resolved. In this regard, it is noteworthy that the triazoles and trifloxystrobin differ relative to their mode of action on eukaryotic cells (Table 1). With triazoles, sterol synthesis is modified via the inhibition of 14α-demethylase, resulting in a loss of plasma membrane integrity (Ghannoum and Rice 1999; Mueller 2006). With trifloxystrobin, mitochondrial respiration is inhibited by fungicidal binding to

Table 3 LD_{50} and %C1/2 values for fungicides and other agrichemicals

Name	LD_{50} rats (mg/kg)[a]	%C1/2 (μM)[b]	Category	Rank order (LD_{50})[c]	Rank order (%C½)[c]
Myclobutanil	1,600	111	Fungicide	10	9
Tebuconazole	4,000	98	Fungicide	13	8
Propiconazole	1,517	44	Fungicide	9	4
Tetraconazole	1,248	39	Fungicide	6	3
Trifloxystrobin	>5,000	0.7	Fungicide	14	1
Oxamyl	5	81	Insecticide	1	7
Chlorpyrifos	135–163	50	Insecticide	4	5
Malathion	1,375	80	Insecticide	8	6
Malaoxon	158	116	Insecticide	3	10
Methyl Parathion	9–25	235	Insecticide	2	12
Alachlor	930–1,350	24	Herbicide	7	2
Atrazine	1,869–3,090	210	Herbicide	12	11
2,4-D	375–666	515	Herbicide	5	13
Dicamba	757–1,707	2247	Herbicide	11	14
Trifluralin	$>10^4$	5936	Herbicide	15	15

[a] Sources for LD_{50} values (Sorensen et al. 2003; Dow Agrosciences 2005; Wagner et al. 2005; U.S. EPA 2006b)

[b] Sources for %C1/2 values: fungicides (this study) and herbicides and insecticides (Sorensen et al. 2003; Wagner et al. 2005)

[c] Rank order based on LD_{50} or %C1/2 values

the Q_0 site on cytochrome *b*, resulting in a loss of ATP synthesis (Bartlett et al. 2002). Thus, it is possible that these fungicides impacted mammalian cells in a dissimilar fashion and that this contributed to the observed differences in cytotoxic effects.

A number of agrichemicals (insecticides and herbicides) have been analyzed in previous studies with the CHO cell microplate cytotoxicity assay and are listed along with the fungicides in Table 3 for comparative purposes. Of the 15 different agents listed, the fungicide trifloxystrobin was the most cytotoxic based on its %C1/2 value of 0.7. This is surprising given that trifloxystrobin has one of the highest LD_{50} values (Table 3) and that strobilurins are generally regarded as safe with minimal risk to human health (Bartlett et al. 2002). A lack of correlation between cytotoxicity and LD_{50} values has been observed by others and has been attributed in part to the differences between single mammalian cell and complex multicellular animal systems (Wagner et al. 2005). Relative to cytotoxicity, the triazole fungicides (myclobutanil, tebuconazole, propiconazole, and tetraconazole) were all ranked, based on %C1/2 values, in the top 10 of the 15 agrichemicals tested (Table 3); their %C1/2 values were essentially in the same range as those reported for the insecticides (except methyl parathion) and the herbicide alachlor, all of which are considered toxic chemicals (U.S. EPA 2006b).

The results of this study demonstrate that a microplate-based assay can be used to quantitatively assess the toxicity of soybean rust fungicides. Moreover, the %C1/2 values measured for these agents may provide some insight into the potential impact that the widespread application of fungicides in response to soybean rust might have on the environment and human health. In this regard, studies are underway to measure the genotoxicity of these agriculturally important chemicals.

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