**Protecting non-target plant species: suggested improvements to current
pesticide registration guidelines.**

By: Andrea L. White, B.Sc.H.

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ABSTRACT

The data generated from current pesticide registration guidelines to predict the effects of herbicides on non-target plant species may be inaccurate and misleading. A series of experiments highlighting different weaknesses in current pesticide registration guidelines were conducted. Results indicated that there is no difference between crop and non-crop species in terms of sensitivity to the herbicides tested. However, variability in sensitivity between crop cultivars, coupled with the ease of germinating non-crop plant species, suggest that wild plant species be included in pesticide registration guidelines. It was also determined that chlorophyll fluorescence, a non-destructive endpoint, is an excellent predictor of early, non-visual herbicidal effects and could provide additional information to risk assessments. Significant differences in phytotoxicity were found between glyphosate and the tank-mix of one of its formulated products indicating that current registration protocol is likely to underestimate herbicide toxicity if testing continues to require data for only the active ingredient.

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INTRODUCTION

Originally, pesticide registration guidelines were implemented to protect plants of economic interest (ie. crops and ornamentals) from the potential damage caused by herbicidal exposure. However, the validity of the data generated from current guidelines to predict the effects of herbicides on non-target plant species and habitats is questionable.

The Pest Management Regulatory Agency (PMRA) in Canada and the Environmental Protection Agency (EPA) in the United States require companies wishing to register a new product to provide phytotoxicity data for the active ingredient responsible for the pesticide's mode of action. Current guidelines require the collection of phytotoxicity data for 10 crop species, four monocotyledons from two families (including *Zea mays*) and six dicotyledons from four families (including *Glycine max* and a root crop) (Holst and Ellwanger, 1982). Data collected for each species include observations regarding plant appearance and development, presence of visual abnormalities, plant height and weight, and the percentage of effect compared to control plants. Similar to guidelines adhered to in North America, the Organization for Economic Cooperation and Development (OECD) outlines a protocol to determine potential herbicide phytotoxicity. However, the OECD requires data for a minimum of only three crop species (one species from three different categories) for the determination of herbicide phytotoxicity to non-target plants (OECD, 1984).

The identification of potential inadequacies has sparked research regarding current pesticide registration guidelines in regards to the protection of non-target plants and habitats. Species selection has been highlighted as a major problem since crop

species and wild plant species differ in many respects, and therefore, perhaps crops should not be relied upon to represent all plant species in terms of herbicide sensitivity (Fletcher, 1988; Freemark et al., 1990; Cole et al., 1993; Boutin et al., 1995; Boutin et al., 2004).

Another weakness in pesticide regulatory guidelines, closely related to species selection, is the inclusion of only one crop cultivar per species tested. Many varieties exist for any given crop species and differences in herbicide sensitivity exist between cultivars (Garcia-Baudin et al., 1990; Burton et al., 1994; Arsenault and Ivany, 1996; Hulting et al., 2001). This range in herbicide tolerance may result in an inaccurate representation of the phytotoxicity of the herbicide tested depending on which cultivar is used during the registration process.

Current testing may also be improved if methods are incorporated that detect early, sub-lethal effects resulting from herbicide exposure that potentially could be long-lasting. The visual rating system that is currently in place is subjective and does not allow for identification of non-visual effects. Chlorophyll fluorescence (an indirect measure of photosynthesis) can be used as an indicator of environmental stress such as herbicide exposure (Juneau and Popovic, 1999) and has been proposed as a method of identifying injury to leaves without the presence of visible symptoms (Bolhàr-Nordenkamp et al., 1989).

The requirement of toxicity data only for the active ingredient and not for the entire pesticide formulation is another potential inadequacy of current regulatory guidelines. Herbicide formulations include many other ingredients that are considered trade secrets and are therefore largely unknown, as well as the addition of various

surfactants and adjuvants. These additional chemicals have been shown to increase the overall toxicity of the herbicide in question to algae, invertebrates, fish, frogs, fruit flies, and mice (Folmar et al., 1979; Sáenz et al., 1997; Mann and Bidwell, 1999; Grisolia et al., 2004; Howe et al., 2004). Few comparisons of the effect of the active ingredients to that of the formulated product have been conducted for plant species. However, it is well known that different chemicals that are added before spray (e.g. surfactants and adjuvants) to commercial formulations, as well as different formulations containing the same active ingredient, can increase phytotoxicity (Sharma and Singh, 2000; Riechers et al., 1995; Molin and Hirase, 2004). Since current regulatory protocol does not require testing of complete formulations, current tests may be inaccurate and misleading in terms of herbicide phytotoxicity.

Community testing using micro- or mesocosms has been suggested as a method that could give insight into species interactions that may occur resulting from herbicide exposure (Kimball and Levin, 1985; Marrs et al., 1991; Landis et al., 1997). Since phytotoxicity testing focuses on endpoint effects on individual species, the impact on populations or ecosystems are likely not represented and therefore investigation of community and population effects is necessary (Greig-Smith, 1992).

Other potential sections of current pesticide registration guidelines that remain to be investigated are: the number of species selected for inclusion in the testing process (Boutin et al., 1995; Boutin and Rogers, 2000), which phenological stage is best suited for making accurate risk assessments, and the extrapolation of laboratory and/or greenhouse results to the field in an attempt to make ecological predictions (Clark et al., 2004; Höllrigl-Rosta and Schulte, 2005).

Not all of the weaknesses outlined above were investigated in this thesis. However, a series of experiments that highlighted four different components of pesticide regulatory guidelines were conducted: 1) non-crop plant species compared to crop species in regards to herbicide sensitivity, 2) differences in cultivar sensitivity to herbicide exposure, 3) investigation of visual rating and chlorophyll fluorescence as useful endpoints, and 4) toxicity of an active ingredient compared to its formulated product. The objective of this research was to determine whether some aspects of current pesticide regulatory guidelines for phytotoxicity testing are adequate for the protection of non-target/non-crop plant species, and to provide suggestions for improvements, if necessary.

CHAPTER 1.

Improving current pesticide registration guidelines in regards to plant species selection: comparing herbicide sensitivity between crops and their wild relatives.

ABSTRACT

Current pesticide registration guidelines were originally implemented to protect plants of economic interest from the potential damage caused from herbicide exposure. In North America, the inclusion of wild plant species in phytotoxicity testing is not required. Determination of pesticide phytotoxicity currently relies on data provided only for crops to assess the potential risk posed to all plant species exposed to herbicides. The hypothesis stating that there is no difference in herbicide sensitivity between crop species and their closest native wild relatives was tested to determine whether crop species are adequate representatives of both domestic and wild plant species in regards to herbicide sensitivity and phytotoxicity testing. Nine pairs of plant species (which included a crop species and a close relative) and five commonly used agricultural herbicides with different modes of action were included in this study. An IC_{25} (defined as the concentration of herbicide resulting in a 25% response compared to controls) was calculated for every species in which an adequate dose-response curve was produced. Kruskal-Wallis tests comparing the IC_{25} of crop and non-crop species for each herbicide showed no significant differences in sensitivity between species types for any of the herbicides tested. However, subtle differences existed between crops and wild species, and so crop species should not be relied upon exclusively in herbicide phytotoxicity testing for the determination of risk posed to all plant species.

INTRODUCTION

The loss of natural habitats due to intensified agriculture and urban encroachment is putting increased emphasis on the importance of conserving field margins, hedgerows, woodlots, riparian habitats, and wetlands. These habitats are important refuges for annual and perennial plant species (Boutin et al., 1994), and often provide food, shelter, and movement corridors for other wildlife in agricultural landscapes (Kleijn and Snoeiijing, 1997; Marshall and Moonen, 2001). It is well known that these habitats are at risk to herbicide exposure, via direct overspray or indirectly due to spray drift from aerial or ground applications, since they are often adjacent to intensively managed crops (Marrs et al., 1989; Marrs et al., 1991; de Snoo and van der Poll, 1999).

Traditionally, phytotoxicity research has focused on the effects of herbicides on non-target crop species. However, since the 1980s, there have been many studies investigating the effects of herbicides on wild plant species and habitats. It has been shown that overall plant abundance, diversity, and composition of communities are negatively affected by exposure to herbicides (Marshall and Birnie, 1985; Freemark and Boutin, 1995; de Snoo, 1997; Jobin et al., 1997; Kleijn and Snoeiijing, 1997; Boutin and Jobin, 1998; de Snoo, 1999).

Since determination of pesticide phytotoxicity essentially relies exclusively on data provided for agronomically important species to assess the potential risk posed to all plants, research investigating the adequacy of current pesticide registration guidelines in protecting non-target wild plant species and habitats has been undertaken. In 1988, Fletcher et al. suggested that the validity of relying on crop species as surrogates for all plant species in phytotoxicity testing should be challenged due to the lack of information

available regarding wild plant species sensitivities. To date, most research is in agreement that species selection is a major weakness, and that both crop and non-crop plant species should be included in herbicide toxicity testing (Greig-Smith, 1992; Cole et al., 1993; Freemark and Boutin, 1994; Boutin et al., 1995; Boutin and Rogers, 2000; Boutin et al., 2004). Perhaps based in whole or in part by these findings, the OECD recently updated its guidelines to include a list of 52 potential non-crop plant species to be used for herbicide toxicity testing (OECD, 2005).

The objectives of this study were to determine whether crop species and wild plant species differ in their sensitivities to herbicides, and whether current pesticide registration guidelines should expand to include non-crop plant species. More specifically, the hypothesis that there is no difference in herbicide sensitivity between crop species and their closest wild relatives native to eastern Ontario, Canada, and that crop species are therefore adequate representatives of both domestic and wild plants in regards to herbicide phytotoxicity testing was tested.

MATERIALS AND METHODS

This experiment was conducted in the greenhouses at the National Wildlife Research Centre and the Nesbitt Biology building located at Carleton University in Ottawa, Ontario, between April 2004 and August 2005.

Herbicides Selected

Five commercial herbicides containing different active ingredients with various modes of action were chosen for testing: atrazine, bentazon, glyphosate, imazethapyr, and

MCPA. Detailed descriptions of each herbicide can be found in Appendix 1. These particular herbicides were selected because they are all commonly used throughout Canada for agricultural, industrial, and/or domestic purposes.

Plant Species Selection and Growth Requirements

Based on current pesticide registration guidelines in Canada and the United States, the 10 crop species most commonly used for phytotoxicity testing (based on the USEPA on-liner database) and their closest native non-crop relatives found in field margin habitats in Eastern Ontario were selected for this study (Table 1-1). Crop species were chosen based on the most commonly used test species listed in the EPA's one-liner database, and the seed availability of the non-crop relatives. All seeds (both crops and wild species) were obtained from commercial seed suppliers. In the case of *R. sativus*, no non-crop relatives could be found to meet all the selection criteria, so *A. syriaca*, was chosen since this species matched the habitat criteria and had similar morphology to the crop species.

All crop species were germinated in moist vermiculite and transplanted into 10 cm plastic pots containing a rich potting mixture within the seven days after germination. All crop species germinated within seven days of seed sowing. Non-crop species varied in their germination requirements and germination period (see Appendix 2). It should be noted that *A. cernuum* was removed from this study due to extremely poor germination. Therefore, *A. cepa* was also omitted from this experiment. Due to the variable germination and survival rate of *H. strumosus*, coupled with its extreme sensitivity to insect predation, testing with this species (and therefore with *H. annuus*) included only

atrazine rather than all five herbicides. *Z. mays* was not exposed to atrazine due to the insensitivity noted for this species during screening tests, along with the technical difficulties experienced with the spray booth when attempting to apply atrazine at very high concentrations.

Experimental Treatments and Protocol

Initially, 21-day screening tests using five doses (three replicates per dose) ranging from 0-100% of the selected herbicide label rate were performed to establish a known sensitivity range for each species to each of the five herbicides. Herbicides were applied using a track spray booth equipped with a TeeJet 8002E flat-fan nozzle, delivering 6.75 ml/m² at 206.84 kPa (de Vries Manufacturing).

After screening tests were complete and the herbicide sensitivity range for each species was determined, a separate definitive test for each species and herbicide was performed. Six doses (including controls) and depending on the species, either five or six replicates per dose were included for each herbicide. Definitive doses were chosen to span the sensitivity range determined from the screening test and followed a geometric progression from lowest to highest dose (see Appendix 3). All plant species were exposed to a one-time application of each herbicide at the 3-6 leaf stage. Plants were randomly assigned a numerical identification tag to prevent bias during measurements. Visual observations were noted for every plant throughout the 28 day test period, and at 28 days after herbicide exposure, all aboveground green plant material was harvested and placed in a forced-air dryer for a minimum of 72 hours at approximately 70°C for biomass determination.

Data Analyses

An IC_{25} (defined as the herbicide dosage that results in a 25% reduction compared to the controls) was calculated for every species and herbicide using biomass at day 28 of the test period. All IC_{25} were calculated using the linear interpolation method for sub lethal toxicity, also known as the inhibition concentration approach (IC_p) as described in the US EPA report EPA/600/4/-89-001 and 001A.

Non-crop species were compared to the crop species included in this study based on the IC_{25} for each species and herbicide. A Kruskal-Wallis test was performed to investigate whether there were significant differences between the IC_{25} of crop and non-crop species for each of the five herbicides tested. The non-parametric Kruskal-Wallis test was used rather than a one-way analysis of variance (ANOVA) because all of the assumptions of the ANOVA could not be met. In some cases, an IC_{25} could not be calculated due to poor dose-response. Therefore, crop and non-crop species pairs were only included in the correlation and the Kruskal-Wallis tests if an IC_{25} existed for both species within any given pair.

EXPERIMENTAL RESULTS

The number of species and species pairs which were included in the final analyses differed for each herbicide based on dose-response and the ability to calculate an IC_{25} for any given species (Table 1-2). For *A. sativa* and *A. syriaca* sprayed with atrazine, 100% mortality was experienced at every dose tested, which was not expected based on screening test responses, and therefore could not be included in the analyses. Also, for several species, despite initial range-finding tests, no effect on biomass was elicited with

the doses tested. In most cases this involved species that showed no response at 100% label rate during screening tests and therefore were already expected to be quite insensitive to the herbicide in question.

Only species pairs were included in the overall comparison (Figure 1-1). Figure 1-1 shows that there is relatively equal distribution around the 1:1 line indicating that crop and non-crop species are relatively equal in their sensitivity to the herbicides tested. The IC_{25} were generally clustered by herbicide as expected based on label rates, however, those associated with MCPA were more variable. Crop species exposed to MCPA showed much more variability in response compared to their non-crop relatives which were more consistent in terms of sensitivity. Also interesting to note from Figure 1-1 (and referring to Table 1-2) is that out of all the species exposed to imazethapyr, the most tolerant species was the crop *T. aestivum*, while its wild relative, *E. canadensis* was the most sensitive.

Kruskal-Wallis tests for each herbicide comparing the IC_{25} of crop and non-crop species showed that there were no significant differences in herbicide sensitivity between species type for any of the herbicides included in this study (Table 1-3; Figure 1-2). Although there were no significant differences found in herbicide sensitivity between the crop and non-crop species tested, for all herbicides except bentazon the crop species had a lower mean IC_{25} than the non-crop species.

Using all the species included in testing, each species was ranked based on its sensitivity to each herbicide; rankings for non-crop species are shown in Table 1-4. It should be noted that in general the monocotyledons tested were generally more tolerant than the dicotyledons. *E. canadensis* was found to be one of the most tolerant species for

all the herbicides tested regardless of species type; *V. americana* was also quite insensitive. Alternatively, *F. virginiana* was found to be one of the top two most sensitive species overall for both atrazine and bentazon.

DISCUSSION

Boutin and Rogers (2000) and Clark et al. (2004) stated that crop species did not consistently differ from non-crop species in regards to herbicide sensitivity. McKelvey et al. (2002) found that crop species were more or equally sensitive compared to non-crop species, and that in general the most sensitive crop species was an acceptable indicator species for the non-crop species tested. It should be noted that this particular study included data from several sources with varying methods and therefore the conclusions made are questionable. Boutin et al. (2004) concluded that testing with 15 non-crop plant species and six herbicides, indicated that overall wild species were more sensitive than crop species most commonly used in regulatory testing as per the EPA one-liner database.

There is much controversy and conflicting results surrounding the topic of species selection for phytotoxicity risk assessment. This experiment builds on the findings of past research by investigating the differences between crop and non-crop species in a way that has never before been investigated. No study has modeled itself as closely after current regulatory guidelines for phytotoxicity testing. Also, crops have never been paired so close taxonomically to a wild relative like they were in this study.

The results of this experiment are cause for the authors to accept the null hypothesis of this study, which states that there is no difference in herbicide sensitivity

between crop and non-crop species. No overall significant difference was found between crop species sensitivity to herbicide exposure and their native wild relatives in this experiment. However, these same data provide much information in regards to subtle differences between crop and non-crop species. For example, the most sensitive species varied between herbicides - in some cases a crop was more sensitive, in others a non-crop species. Also, individual species sensitivity was quite variable between different herbicides.

Species sensitivity depended on the herbicide applied. In general, it was shown that any given species may be sensitive to one herbicide and relatively tolerant to another. The large variability in plant response among herbicides has been previously reported in the literature (Marshall and Birnie, 1985; Marrs et al., 1989; Marrs et al., 1991; Boutin et al., 2004). This herbicide dependent sensitivity is likely due to many factors such as herbicide mode of action, the ability of a species to metabolize certain chemicals more effectively than others, the efficacy and selectivity of a particular herbicide, etc.

Current guidelines are much too rigid in terms of species requirements, often insisting that crops that are known to be tolerant of the herbicide in question be included as a test species (Table 1-5). For example, bentazon is effective on broadleaf species only. In this study the monocot species, regardless of species type, and soybean and its wild relative *V. americana*, were quite insensitive to bentazon. This was not surprising based on the known selectivity of this herbicide and the fact that it is commonly used on soybean fields to control weeds. Similar trends were noted for atrazine and imazethapyr in that the crops it is commonly used on (mainly corn and soybeans, respectively) and their non-crop relatives were insensitive to these herbicides. As found in this experiment

and reported by Fletcher et al. (1985), corn and soybean can be very tolerant to herbicide exposure; especially corn to atrazine, and soybean to bentazon and imazethapyr. However, it is currently required that both of these species be included in herbicide phytotoxicity tests regardless of their level of tolerance to a given chemical. It was also found in this experiment, and others (Boutin and Rogers; 2000; McKelvey et al., 2002) that grass species are in general more tolerant of herbicide exposure than were broadleaf species. Boutin and Rogers (2000) also stated that monocotyledons exhibited a more consistent response among species than dicotyledons, although this was not necessarily the case in this study with monocot variability differing depending on the herbicide tested.

Knowing that herbicide-dependent variability exists among species, it is suggested that when selecting species for inclusion in regulatory testing those that are known to be insensitive or those that the herbicide is intended for use should not be included. Ideally, guidelines would allow the selection of species included to be tailored to a specific herbicide or even region/habitat in which the herbicide may be utilized to better protect species of conservation interest as well as agronomically important species.

Wild plant species are often thought to be much more difficult to grow and more variable in general than crop plant species which are purposely bred to produce fast-growing, equal growth stage monocultures. All of the species included in this paper were relatively easy to germinate and maintain, and none of these species were chosen based on those criteria, which suggests that non-crop species are not that much more difficult to than their crop relatives. In particular, all of the grass species included in this study and the broadleaf species *F. virginiana*, *S. nigrum*, and *L. canadensis*, are excellent species to

work with, requiring little or no special treatment compared to crop species germination and growth requirements. There are also many examples in the primary literature of wild plant species being easily utilized for herbicide phytotoxicity testing (see OECD, 2005).

It has been identified so far that there are species- and herbicide-dependent differences in sensitivity, including differences between species types as well as between monocotyledons and dicotyledons. It is known that differences in sensitivity exist between cultivars of the same crop species (Garcia-Baudin, 1990; Burton et al., 1994; Arsenault and Ivany, 2001; Hulting et al., 2001; White and Boutin, 2005). If crop species are to be included in pesticide regulatory guidelines, this variability should be acknowledged and perhaps several varieties of the same crop species should be included in phytotoxicity testing. What is not known, however, is whether differences in wild species exist between species from different ecotypes, geographic regions, or environmental conditions. Similar variability may exist between wild species populations as it does between crop cultivars and should be investigated further.

CONCLUSION

In conclusion, there are many uncertainties associated with species selection and herbicide regulatory guidelines. No significant difference in herbicide sensitivity was discovered between the crop species and their non-crop relatives included in this study. However, it is suggested based on the results found in this experiment, that crop species in which a specific herbicide is intended for use, as well as those with known tolerance, should not be included in phytotoxicity testing. Based on equally variable species-dependent response between crops and non-crops, not to mention monocotyledons and dicotyledons, the differences between cultivars of crop species, and the ecological importance of many wild plant species, current pesticide registration guidelines should include non-crop plant species. It should also be reiterated that most non-crop species do not require significantly more attention in regards to germination and growth requirements than crop species.

Table 1-1. Plant species chosen for phytotoxicity testing. Crop species (C) are shown paired with their non-crop (NC) relative.

Species	Species Type	English Common Name	Family
<i>Allium cepa</i>	C	Onion var. Spanish Sweet Utah	Liliaceae
<i>Allium cernuum</i>	NC	Nodding Onion	Liliaceae
<i>Avena sativa</i>	C	Oats var. AC Stewart	Poaceae
<i>Poa compressa</i>	NC	Canada Bluegrass	Poaceae
<i>Triticum aestivum</i>	C	Wheat var. AC Brio	Poaceae
<i>Elymus canadensis</i>	NC	Northern Wheatgrass	Poaceae
<i>Zea mays</i>	C	Corn var. Seneca Chief	Poaceae
<i>Andropogon gerardii</i>	NC	Big Bluestem	Poaceae
<i>Fragaria ananassa</i>	C	Strawberry (F1 Hybrid)	Rosaceae
<i>Fragaria virginiana</i>	NC	Thick-leaved Strawberry	Rosaceae
<i>Glycine max</i>	C	Soybean var. Maple Glen	Fabaceae
<i>Vicia americana</i>	NC	American Vetch	Fabaceae
<i>Helianthus annuus</i>	C	Sunflower var. Mammoth Russian	Asteraceae
<i>Helianthus strumosus</i>	NC	Rough-leaved Sunflower	Asteraceae
<i>Lactuca sativa</i>	C	Lettuce var. Tom Thumb	Asteraceae
<i>Lactuca candensis</i>	NC	Tall Blue Lettuce	Asteraceae
<i>Raphanus sativus</i>	C	Radish var. Minowase	Brassicaceae
<i>Asclepias syriaca</i>	NC	Common Milkweed	Asclepiadaceae
<i>Solanum lycopersicon</i>	C	Tomato var. Beefsteak	Solanaceae
<i>Solanum nigrum</i>	NC	Black Nighshade	Solanaceae

Table 1-2. The IC₂₅ calculated for each species where possible (with confidence intervals shown in brackets).

Herbicide	Crop Species	IC ₂₅ (g ai/ha)	Non-crop Species	IC ₂₅ (g ai/ha)
Atrazine	<i>A. sativa</i>	100% kill ^a	<i>P. compressa</i>	71 (40, 88)
	<i>T. aestivum</i>	148 (118, 246)	<i>E. canadensis</i>	217.08 (n/a)
	<i>Z. mays</i>	insensitive ^b	<i>A. gerardii</i>	2162 (n/a)
	<i>F. ananassa</i>	99 (n/a)	<i>F. virginiana</i>	16 (1, 26)
	<i>G. max</i>	165 (119, 240)	<i>V. americana</i>	525 (253, 712)
	<i>H. annuus</i>	72 (66, 84)	<i>H. strumosus</i>	63 (36, 96)
	<i>L. sativa</i>	40 (35, 49)	<i>L. canadensis</i>	97 (n/a)
	<i>R. sativus</i>	79 (72, 91)	<i>A. syriaca</i>	100% kill ^a
	<i>S. lycopersicon</i>	55 (52, 59)	<i>S. nigrum</i>	67 (52, 131)
Bentazon	<i>A. sativa</i>	no effect ^c	<i>P. compressa</i>	no effect ^c
	<i>T. aestivum</i>	3066 (n/a)	<i>E. canadensis</i>	no effect ^c
	<i>Z. mays</i>	no effect ^c	<i>A. gerardii</i>	3708 (n/a)
	<i>F. ananassa</i>	515 (245, 784)	<i>F. virginiana</i>	85 (57, 146)
	<i>G. max</i>	no effect ^c	<i>V. americana</i>	1095 (584, 2985)
	<i>H. annuus</i>	not tested ^d	<i>H. strumosus</i>	not tested ^d
	<i>L. sativa</i>	310 (283, 339)	<i>L. canadensis</i>	142 (106, 224)
	<i>R. sativus</i>	78 (69, 87)	<i>A. syriaca</i>	258 (208, 501)
	<i>S. lycopersicon</i>	1272 (764, 2284)	<i>S. nigrum</i>	519 (385, 1660)

Herbicide	Crop Species	IC25 (g ai/ha)	Non-crop Species	IC25 (g ai/ha)
Glyphosate	<i>A. sativa</i>	54 (48, 63)	<i>P. compressa</i>	27 (13, 35)
	<i>T. aestivum</i>	20 (15, 28)	<i>E. canadensis</i>	84 (64, 91)
	<i>Z. mays</i>	92 (73, 111)	<i>A. gerardii</i>	62 (n/a)
	<i>F. ananassa</i>	35 (n/a)	<i>F. virginiana</i>	70 (66, 75)
	<i>G. max</i>	67 (35, 87)	<i>V. americana</i>	111 (87, 159)
	<i>H. annuus</i>	not tested ^d	<i>H. strumosus</i>	not tested ^d
	<i>L. sativa</i>	112 (94, 155)	<i>L. canadensis</i>	115 (76, 143)
	<i>R. sativus</i>	101 (86, 112)	<i>A. syriaca</i>	36 (30, 56)
	<i>S. lycopersicon</i>	51 (27, 74)	<i>S. nigrum</i>	76 (63, 105)
Imazethapyr	<i>A. sativa</i>	2.6 (2.4, 2.9)	<i>P. compressa</i>	18 (17, 20)
	<i>T. aestivum</i>	3 (2, 5)	<i>E. canadensis</i>	51 (21, 82)
	<i>Z. mays</i>	10 (8, 13)	<i>A. gerardii</i>	4 (n/a)
	<i>F. ananassa</i>	3 (2, 4)	<i>F. virginiana</i>	4.5 (4.1, 5.2)
	<i>G. max</i>	no effect ^c	<i>V. americana</i>	no effect ^c
	<i>H. annuus</i>	not tested ^d	<i>H. strumosus</i>	not tested ^d
	<i>L. sativa</i>	no effect ^c	<i>L. canadensis</i>	7 (n/a)
	<i>R. sativus</i>	7 (n/a)	<i>A. syriaca</i>	17 (16, 20)
	<i>S. lycopersicon</i>	15 (13, 21)	<i>S. nigrum</i>	3 (2, 6)

Herbicide	Crop Species	IC25 (g ai/ha)	Non-crop Species	IC25 (g ai/ha)
MCPA	<i>A. sativa</i>	432 (334, 632)	<i>P. compressa</i>	304 (265, 352)
	<i>T. aestivum</i>	489 (403, 683)	<i>E. canadensis</i>	1637 (n/a)
	<i>Z. mays</i>	325 (261, 430)	<i>A. gerardii</i>	690 (422, 2393)
	<i>F. ananassa</i>	68 (46, 148)	<i>F. virginiana</i>	92 (84, 103)
	<i>G. max</i>	67 (53, 122)	<i>V. americana</i>	no effect ^c
	<i>H. annuus</i>	not tested ^d	<i>H. strumosus</i>	not tested ^d
	<i>L. sativa</i>	6 (5, 8)	<i>L. canadensis</i>	44 (33, 81)
	<i>R. sativus</i>	7 (6, 7)	<i>A. syriaca</i>	126 (112, 159)
	<i>S. lycopersicon</i>	50.51 (29, 83)	<i>S. nigrum</i>	224 (200, 266)

a: herbicide doses resulted in 100% mortality despite range-finding test

b: this species not tested due to extreme insensitivity exhibited during screening tests, as well as technical difficulties that result when spraying atrazine at high concentrations

c: herbicide doses did not elicit a response that resulted in a 25% effect compared to controls

d: these species were not tested due to poor germination

Table 1-3. Summary of results from Kruskal-Wallis tests investigating differences between crop and non-crop species for each herbicide. It should be noted that only pairs in which both species had an IC₂₅ were included in analysis.

Herbicide	Label Rate (g ai/ha)	Sample Size	Chi-square	Degrees of Freedom	Asymp. Sig.	Mean Crop IC₂₅ (g ai/ha)	Std. Error (g ai/ha)	Mean Non- Crop IC₂₅ (g ai/ha)	Std. Error (g ai/ha)
Atrazine	1296	12	0.026	1	0.873	96.4	20.7	164.2	77.4
Bentazon	960	8	0.333	1	0.564	543.7	258.8	251.0	96.3
Glyphosate	2136	14	0.276	1	0.600	66.4	11.5	72.6	11.1
Imazethapyr	91.2	10	1.641	1	0.200	20.6	14.5	26.1	10.1
MCPA	750	16	0.918	1	0.338	196.7	79.8	445.2	214.8

Table 1-4. Non-crop species sensitivity ranking based on total species tested for each herbicide. The lower the number, the more sensitive the species is to each herbicide.

Species	Atrazine n = 15	Bentazon n = 12	Glyphosate n = 16	Imazethapyr n = 14	MCPA n = 16
Monocotyledons					
<i>A. gerardii</i>	15	11	7	5	14
<i>E. canadensis</i>	13	12	11	13	15
<i>P. compressa</i>	6	12	2	12	3
Dicotyledons					
<i>A. syriaca</i>	---	4	4	11	10
<i>F. virginiana</i>	1	2	9	6	9
<i>H. strumosus</i>	4	---	---	---	---
<i>L. canadensis</i>	9	3	16	8	4
<i>S. nigrum</i>	5	7	10	4	11
<i>V. americana</i>	14	8	14	14	16

Table 1-5. Examples from this study to support the idea that species with a known tolerance to a certain herbicide, as well as those in which the herbicide is intended for use, not be included in phytotoxicity risk assessment for that herbicide. Monocots = monocotyledons; Dicots = dicotyledons.

Herbicide	Crops on which the herbicide can be used	Type of species the herbicide is effective at controlling	Species from this study exhibiting tolerance
Atrazine	<i>Z. mays</i>	Mainly dicots	<i>Z. mays</i> <i>A. gerardii</i>
Bentazon	<i>Z. mays</i> <i>G. max</i> Forage grasses	Dicots only	<i>Z. mays</i> <i>G. max</i> <i>V. americana</i> All other monocots tested
Imazethapyr	<i>G. max</i>	Monocots and dicots	<i>G. max</i> <i>V. americana</i>

Figure 1-1. Comparison between crop species and their closest native wild relatives for five different herbicides.

Figure 1-2. Variability in herbicide sensitivity between crop and non-crop species for five different commercial herbicides.

Figure 1-1.

Figure 1-2.

CHAPTER 2.

Investigating variability in herbicide sensitivity among cultivars of five different crop species: implications for risk assessment.

ABSTRACT

During the registration process, risk assessment of potential herbicidal effects on wild vegetation is conducted using crop species. For any given crop species, there are many cultivars, some of which may exhibit varying levels of sensitivity to a particular herbicide. This range in herbicide tolerance may result in an inaccurate representation of the phytotoxicity of the herbicide being tested depending on which cultivar is included. The objective of this study was to determine whether current pesticide registration guidelines should be expanded to include phytotoxicity data for several cultivars of the same crop species. Several cultivars of five crop species (onion, lettuce, radish, corn, and tomato) were exposed to three commercial herbicides containing atrazine, MCPA amine, and imazethapyr. All cultivars of each of the crops tested exhibited varying degrees of sensitivity to the herbicides tested. Cultivar sensitivity was not only variable, but also herbicide dependent. These results suggest that pesticide registration guidelines should acknowledge the differences in cultivar sensitivity to herbicides and require the inclusion of phytotoxicity data for several cultivars of the same crop.

INTRODUCTION

Pesticide registration guidelines were originally implemented to protect plants of economic interest (ie. crops and ornamentals) from the potential damage caused by

accidental herbicide exposure. Currently in North America, pesticide regulatory agencies require companies wishing to register any herbicide to provide phytotoxicity data for 10 different crop species, four monocots and six dicots (including *Zea mays* and *Glycine max*) (Holst and Ellwanger, 1982). Originally, the Organization of Economic Development (OECD) required only three crop species (one species from three different categories) to determine herbicide phytotoxicity to non-target plants (OECD, 1984). Recently, the OECD updated their pesticide registration guidelines to include a list of 52 potential non-crop plant species to be used for herbicide toxicity testing (OECD, 2005). However, determination of pesticide phytotoxicity has traditionally relied on data provided for agronomically important species to determine the potential risk posed to both crop and non-crop plant species.

Based on the experimental data collected to date, it is obvious that non-target flora surrounding agricultural fields is significantly at risk from herbicide drift (Marrs et al., 1989; Marrs et al., 1991; Kleijn and Snoeijs, 1997; de Snoo and van der Poll, 1999; Follak and Hurle, 2003), and also that current pesticide registration guidelines may not be adequate for the protection of non-target vegetation and natural habitats (Freemark et al., 1990; Greig-Smith, 1992; Cole et al., 1993; Freemark and Boutin, 1994; Boutin et al., 2004). A major weakness in current guidelines is the emphasis on testing crop plant species, whose phytotoxic responses are used to determine the sensitivity of all non-target plant species (including non-crop plants) to herbicide exposure.

For any given crop species, there are many different cultivars, some of which may exhibit varying levels of sensitivity to a particular herbicide. It is known that levels of herbicide tolerance differ among cultivars of various crop species; for example: corn

(Keifer, 1989; Rowe et al., 1990; Burton et al., 1994), soybean (Wax et al., 1976; de Weese et al., 1989; Dayan et al., 1997; Hulting et al., 2001), potato (Graf and Ogg, 1976; Friesen and Wall, 1984; Arsenault and Ivany, 1996; Arsenault and Ivany, 2001), several kinds of beans (Bauer et al., 1995; Urwin et al., 1996), cotton (Abernathy et al., 1979; Molin and Khan, 1996), wheat (Bacon et al., 1986; Garcia-Baudin et al., 1990), peppers (Schroeder, 1992), pumpkins (Harrison and Keinath, 2003), cabbage (Hopen et al., 1993), collard and kale (Harrison et al., 1998), and peas (Jensen, 1993). This range in herbicide tolerance may result in an inaccurate representation of the phytotoxicity of the herbicide being tested depending on which cultivar is used during the registration process.

Based on the null hypothesis that all cultivars of a particular crop exhibit equal sensitivity to any given herbicide, the objective of this study was to determine whether current pesticide registration guidelines for phytotoxicity testing should be expanded to include several different cultivars of the same crop species. It should be emphasized that the purpose of this study was to determine if the differences in herbicide sensitivity between crop cultivars were large enough to warrant a re-evaluation of current risk assessment protocols, unlike past research which has aimed to establish the differences in cultivar sensitivity to herbicide exposure solely for the determination of crop margins of safety for agronomic benefit.

MATERIALS AND METHODS

This experiment was conducted in the greenhouses at the National Wildlife Research Centre located at Carleton University in Ottawa, Ontario, between March 2004 and April 2005.

Herbicides Selected

Three commercial agricultural herbicides, Aatrex Liquid 480, Pursuit, and MCPA Amine 500, containing atrazine, imazethapyr, and MCPA amine, respectively, were selected for testing. Detailed descriptions of each herbicide can be found in Appendix 1. Each of the herbicides chosen are commonly used in the Great Lakes – St. Lawrence basin region of Canada on major crop species for pre- and/or post-emergence control of herbaceous and grassy weed species.

Crop Species and Cultivar Selection

All crops included in this study are commonly used in regulatory testing for the determination of herbicide toxicity to non-target plant species. Efforts were made to randomly choose cultivars that represented a range of different uses/characteristics for each crop species tested. Availability of seeds from local seed suppliers was also a factor when selecting cultivars for inclusion in this experiment.

Onion (*Allium cepa*), lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), tomato (*Solanum lycopersicon*), and corn (*Zea mays*) were selected for herbicide sensitivity testing, with each crop species (except onion) represented by five different cultivars with unique characteristics (Table 2-1). Only three onion cultivars were tested.

Plant Growth Conditions and Experimental Protocol

Seeds were germinated in moist vermiculite and transplanted into 10 cm plastic pots containing a rich potting mixture within seven days of germination. During the test period, temperatures ranged between 15 - 26°C with a 16-hour photoperiod

(supplemented with artificial light when necessary) and watered when required. The predatory mite, *Amblyseius cucumeris*, was applied weekly to lettuce, radish, and tomato plants to control minor thrips (several different species) infestations.

Initially, 21-day range-finding tests using five doses (three replicates per dose) ranging from 1-100% of the label rate were performed to determine the sensitivity range to each herbicide for each crop using a randomly selected cultivar. Herbicides were applied using a track spray booth equipped with a TeeJet 8002E flat-fan nozzle, delivering 6.75 ml/m² at 206.84 kPa (de Vries Manufacturing). Chemical analyses of the sprayed solutions showed that close to 100% of the intended doses was sprayed on the plants.

Once range-finding tests were complete, a definitive test for each crop and cultivar, using six doses (including controls) with five replicates per dose, was performed for each herbicide. Definitive test doses were chosen to span the sensitivity range determined in the screening test and followed a geometric progression from lowest to highest dose (Appendix 3). If during the screening test a species showed no response after exposure to any herbicide at 100% label rate, the doses chosen for the definitive test ranged from 100-506% of the herbicide label rate. Broadleaf crops were exposed to a one-time herbicide application at the 3-5 leaf stage, with onion and corn being sprayed at the 2-4 leaf stage.

No definitive test was performed for corn with atrazine due to the insensitivity (no effect at 100% label rate) of this crop. Doses of atrazine, when greater than the label rate, result in application problems due to the thick consistency of this herbicide formulation.

Visual observations were noted for every plant before herbicide application as well as 14 days after spray. At 28 days after herbicide exposure, visual observations were recorded and all aboveground green plant material was harvested and placed in a forced-air dryer for 72 hours at approximately 70°C for biomass determination.

Data Analyses

To account for differences in size between cultivars, the total percent of control based on plant biomass, for each crop cultivar and herbicide was determined. A two-way analysis of variance, comparing the total percent control to herbicide dose and cultivar, was performed for each crop to distinguish whether there were significant differences in herbicide sensitivity between the cultivars tested. The assumptions of the two-way analysis of variance were met for all species. However, *A. cepa* (exposed to atrazine and MCPA) only loosely met the assumptions due to the erratic response of this species.

An IC_{50} (defined as the effective dosage that results in a 50% reduction in biomass) was calculated for every cultivar and herbicide. All IC_{50} were calculated using the linear interpolation method for sub lethal toxicity, also known as the inhibition concentration approach (IC_p) as described in the US EPA report EPA/600/4/-89-001 and 001A. If a cultivar of any species did not show a 50% reduction in biomass when compared to the control or if there was 100% mortality, twice the highest dose or half the lowest dose included in the definitive test was used as the IC_{50} , respectively.

EXPERIMENTAL RESULTS

For plants exposed to atrazine and MCPA amine, significant differences were revealed between cultivars for all species; plants exposed to imazethapyr exhibited significant differences between cultivars for all species except *R. sativus* (Table 2-2).

Relative to the other species studied here, *A. cepa* cultivars were generally insensitive to atrazine, especially the variety Spanish Sweet Utah which exhibited dramatic hormesis in response to sub-lethal herbicide exposure (Figure 2-1). Variability within cultivar was especially high for the *A. cepa* variety White Pickling, as well as the *R. sativus* varieties Cherry Belle and French Breakfast exposed to atrazine (Figure 2-1). All species with sensitive cultivars (ie. all species except *A. cepa*) exposed to atrazine suffered from withering, necrosis, and mottled pigmentation by 14 days after herbicide exposure.

Differences among cultivars exposed to imazethapyr were not as obvious for all species compared to the other herbicides tested and good dose-response relationships were rarely produced for any of the species included in this study (Figure 2-2). Again, *A. cepa* exhibited great within cultivar variability for the varieties Ishikuro and Spanish Sweet Utah (Figure 2-2). Visual effects associated with exposure to imazethapyr were generally a reduction in biomass. However, at doses close to or greater than the label rate, leaves became leather-like and darker in colour.

Excellent dose-response curves were produced for cultivars of most species exposed to MCPA amine (Figure 2-3). Again, *A. cepa* exhibited within cultivar variability for varieties Ishikuro and Spanish Sweet Utah. Some cultivars of *L. sativa*, *R. sativus*, and *Z. mays* showed hormesis after MCPA amine exposure (Figure 2-3). *Z. mays*

is quite insensitive to MCPA amine, with minimal effects occurring at up to five times the field label rate, although dose-response relationships were obvious for all cultivars tested (Figure 2-3). Visual effects such as leaf and stem bending and twisting, along with stem malformation were quite dramatic for all species sprayed with MCPA amine as early as one day after spray (except *Z. mays*, for which visual effects did not occur for nearly one week).

A scatterplot of the $\log IC_{50}$ (g ai/ha) for each of the crop cultivars tested illustrates the wide range in sensitivity among crop varieties, as well as between herbicides (Figure 2-4). The IC_{50} (g ai/ha) for each cultivar of each species for all herbicides tested can be found in Table 2-3. For species sprayed with atrazine, *R. sativus* had the least variability between cultivars, with the IC_{50} ranging from 32 – 227 g ai/ha; *A. cepa* was the most variable with the IC_{50} ranging between 190 – 1115 g ai/ha for the cultivars tested. Regarding imazethapyr, Figure 2-4 may be misleading because for *R. sativus*, no 50% effect was achieved for any of the cultivars with the doses of imazethapyr tested so the IC_{50} was estimated to be twice the highest dose that the plants were exposed and therefore it appears as if there was no cultivar variability. If *R. sativus* is excluded, *A. cepa* has the least variability among cultivars with the IC_{50} ranging from 1.7 – 2.3 g ai/ha. The crop exposed to imazethapyr showing the most variability among cultivars was *Z. mays*, with the IC_{50} ranging from 12 – 202 g ai/ha. For crops sprayed with MCPA, *S. lycopersicon* was the crop showing the least amount of variability among its cultivars (IC_{50} ranging from 39 – 73 g ai/ha). *Z. mays* showed the greatest variability with the IC_{50} ranging from 310 – 7590 g ai/ha.

The range in tolerance to the herbicides tested was compared using the IC_{50} of the most and least sensitive cultivar for each species (Table 2-4). For atrazine, if the most sensitive cultivar of each species was chosen, the herbicide tolerance ranged from approximately 1 – 15% label rate. This is much less than if the least sensitive cultivar of each species was chosen, where the range in sensitivity is ~ 13 – 86% of the label rate. For imazethapyr, notable differences in herbicide tolerance were encountered depending on whether the most or least sensitive cultivars were chosen, about 2% to two times greater than the label rate, and approximately 2.5% to ten times the label rate, respectively. Similar results were found for MCPA Amine, with the IC_{50} s ranging from about 1.5 – 41% of the label rate for the most sensitive cultivars, and from 8% to 10 times greater than the label rate if the cultivars exhibiting the least sensitive tolerance are chosen.

DISCUSSION

Unlike previous research investigating herbicide sensitivity differences among crop cultivars for agronomic benefit, this project emphasized utilizing the knowledge of this variability to improve risk assessment for conservation purposes. However, the methodology used in this experiment was not unlike other studies investigating differences in herbicide sensitivity among crop cultivars.

All cultivars of each of the crop species included in this experiment exhibited varying degrees of sensitivity to the three herbicides tested. The variability among cultivars, as well as species, is not surprising based on past research, which aimed to determine margins of safety for different crop species to various herbicides. However, it

was shown in this study that depending on the cultivar chosen, different conclusions about the phytotoxicity of a given herbicide may differ.

Harrison and Keinath (2003) found a wide range of herbicide sensitivity among 67 commercial and experimental pumpkin cultivars exposed to clomazone; Keifer (1989) and Hopen et al. (1993) also found that clomazone sensitivity varied among both corn and cabbage cultivars, respectively. Potato cultivars ranged in their level of sensitivity to metribuzin (Graf and Ogg, 1976; Friesen and Wall, 1984; Aresnault and Ivany, 1996; Aresnault and Ivany, 2001), as did soybeans (Wax et al., 1976; de Weese et al., 1989). Varietal differences have also been noted for soybeans exposed to sulfentrazone (Hulting et al., 2001) and sweet cherries exposed to chlorsulfuron (Bhatti et al., 1995).

The data collected in this experiment revealed that cultivar sensitivity is not only variable, but also herbicide dependent. For example, the cultivar Grand Rapids of *L. sativa* was the most sensitive to atrazine while All Year Round was more sensitive to MCPA amine and imazethapyr. Similarly, Tiny Tim of *S. lycopersicon* was the most sensitive cultivar to atrazine while Amish Paste was by far the most sensitive cultivar when sprayed with imazethapyr. These results agree with Abernathy et al. (1979) who compared 48 cotton cultivars exposed to atrazine and propazine and found that one of the most sensitive cultivars to atrazine was among the most tolerant to propazine. Urwin et al. (1996) also noted that the most and least tolerant dry edible bean (*Phaseolus vulgaris*) cultivar differed among four different herbicides. This herbicide-specific variation between both cultivars and species results in a low predictive ability when attempting to make accurate broad ecological risk assessments.

This study did not attempt to explain why the differences in herbicide sensitivity between cultivars or crop species were occurring. However, the idea that differences in the absorption, translocation, and metabolism of herbicide could be responsible for cultivar/species variation in regards to herbicide sensitivity has been investigated (Rowe et al., 1990; Burton et al., 1994; Bauer et al., 1995; Molin and Khan, 1996; Dayan et al., 1997). Environmental factors such as soil type and temperature, when coupled with the application of metribuzin, have also been shown to result in differences in yield among pepper cultivars (Schroeder, 1992). However, it can be assumed that factors such as soil type, light conditions, watering regimes, and to some extent temperature, did not affect the results of this experiment since all plants were grown in the same greenhouse conditions.

Currently in risk assessment, the most sensitive species of all the species tested is chosen to determine what dose of a specific herbicide would pose the least risk to non-target vegetation. For crop species, depending on which cultivar has been chosen to be included in the testing process, very different recommendations may be made.

In this study notable differences in herbicide tolerance have been found between cultivars of the same crop species. Based on the response of the crop cultivars included in this experiment, prediction of what is a harmful herbicide dose can range from approximately 1 – 13% of the herbicide label rate depending on whether the cultivar chosen for risk assessment is quite tolerant or quite sensitive to a specific herbicide, in this case for atrazine. This range in cultivar sensitivity, coupled with the supposed arbitrary selection of cultivars for risk assessment could alter the outcome of the risk assessment conducted during the regulatory process, and ultimately pose a threat to non-

target vegetation via herbicide drift. It has been found that drift can contain extremely small amounts (< 1%) or up to 40 % of the herbicide label rate (Norby and Skuterud, 1975; Bode et al., 1976; Byass and Lake, 1977; Maybank et al., 1978; Marrs et al., 1989; Longley et al., 1997). However the accepted value for drift varies between 5% and 10% depending on the conditions and mode of herbicide spray. The range in potential drift, combined with the variability among cultivar sensitivity makes it necessary to choose crop cultivars that adequately reflect the sensitivity of non-target plant species.

CONCLUSION

Development of a new herbicide requires research investigating its phytotoxicity to the crop species on which it will be used for weed control, as well as those crops that may be exposed accidentally (e.g. drift, direct overspray, etc.). This study shows that there is a great potential for variation among different cultivars of the same crop species, and that these differences are large enough to alter the results of a risk assessment depending on the cultivar included in testing. Therefore, it is suggested that current regulatory guidelines for North America, as well as those provided by the OECD and the European community, should require the testing of multiple crop varieties when determining herbicide phytotoxicity. Alternatively, the provision of the plant screening data collected when determining herbicide efficacy and crop margin of safety prior to herbicide registration would assist in determining the sensitivity of cultivars for any given crop (Boutin and Rogers, 2000). In many cases depending on the cultivar tested, very different conclusions could be made regarding the toxicity of a specific herbicide.

Table 2-1. Overview of the different cultivars of each crop species selected for testing.

Crop Species	Crop Variety	Unique Variety Characteristics
<i>Allium cepa</i>	var. Ishikuro var. Spanish Sweet Utah var. White Pickling	- spring onion; does not form bulbs - large globe variety with mild taste - small, round, white onions used for pickling
<i>Lactuca sativa</i>	var. All Year Round var. Cassandra var. Grand Rapids var. Tom Thumb var. Triumph	- drought resistant; hardy and reliable - resistant to downy mildew and Lettuce Mosaic Virus - heritage variety - dwarf variety - iceberg variety; root aphid tolerance
<i>Raphanus sativus</i>	var. Cherry Belle var. China Rose var. French Breakfast var. Minowase var. Sparkler White Tip	- early variety; cherry red root; mild flavour - late variety; long, cylindrical, pink root - heritage variety; sweet flavour - large, white root - round root with white tip
<i>Solanum lycopersicon</i>	var. Amish Paste var. Beefsteak var. Long Keeper var. Tiny Tim var. Vendor	- heritage variety - high yielding; large fruit - long term storage variety - dwarf variety - Tomato Mosaic Virus and verticillium resistant
<i>Zea mays</i>	var. DK427 var. 38T28 (Bt) var. Golden Bantam var. Robust var. Seneca Chief	- conventional variety - Bt resistant - heritage variety - popcorn variety - sweet corn

Table 2-2. Summary of p-values from the two-way ANOVAs comparing percent of control to variety as well as dose for each of the species tested with three different herbicides. For each herbicide and species, n = 25.

Herbicide	Species	Variety	Dose
Atrazine	<i>Allium cepa</i>	0.001	0.503
	<i>Lactuca sativa</i>	< 0.001	< 0.001
	<i>Raphanus sativus</i>	0.016	< 0.001
	<i>Solanum lycopersicon</i>	< 0.001	< 0.001
Imazethapyr	<i>Allium cepa</i>	0.009	0.027
	<i>Lactuca sativa</i>	< 0.001	0.055
	<i>Raphanus sativus</i>	0.114	0.096
	<i>Solanum lycopersicon</i>	< 0.001	0.117
	<i>Zea mays</i>	< 0.001	0.025
MCPA	<i>Allium cepa</i>	0.003	0.022
	<i>Lactuca sativa</i>	< 0.001	< 0.001
	<i>Raphanus sativus</i>	< 0.001	< 0.001
	<i>Solanum lycopersicon</i>	< 0.001	< 0.001
	<i>Zea mays</i>	< 0.001	< 0.001

Table 2-4. The IC_{50} calculated for each cultivar of the five different crop species tested (with confidence intervals shown in brackets). ** corresponds to values that were estimated to be twice the applied dose due to lack of 50% response; * corresponds to values that were estimated to be half the lowest applied dose due to 100% mortality.

Herbicide	Species	Cultivar	IC_{50} (g ai/ha)
Atrazine	<i>Allium cepa</i>	Ishikuro	1115**
		Spanish Sweet Utah	1115**
		White Pickling	190 (n/a)
	<i>Lactuca sativa</i>	All Year Round	164 (91, 226)
		Cassandra	43 (27, 120)
		Grand Rapids	15 (12, 24)
		Tom Thumb	114 (73, 132)
		Triumph	76 (43, 116)
		Cherry Belle	184 (41, 300)
		China Rose	62 (38, 268)
	<i>Raphanus sativus</i>	French Breakfast	227 (82, 327)
		Minowase	32 (21, 51)
		Sparkler	40 (32, 66)
	<i>Solanum lycopersicon</i>	Amish Paste	155 (115, 203)
		Beefsteak	226 (172, 248)
		Long Keeper	236 (194, 249)
		Tiny Tim	17 (13, 27)
		Vendor	250 (168, 308)
Imazethapyr	<i>Allium cepa</i>	Ishikuro	2.3 (1.9, 2.8)
		Spanish Sweet Utah	2 (1.7, 2.7)
		White Pickling	1.7 (1.6, 1.8)
	<i>Lactuca sativa</i>	All Year Round	208 (69, 344)
		Cassandra	923**
		Grand Rapids	923**
		Tom Thumb	923**
		Triumph	923**
	<i>Raphanus sativus</i>	Cherry Belle	16**
		China Rose	16**
		French Breakfast	16**
		Minowase	16**
		Sparkler	16**

Herbicide	Species	Cultivar	IC ₅₀ (g ai/ha)
Imazethapyr	<i>Solanum lycopersicon</i>	Amish Paste	23*
		Beefsteak	9 (8, 11)
		Long Keeper	10 (8, 12)
		Tiny Tim	9 (8, 9)
		Vendor	13 (11, 19)
	<i>Zea mays</i>	38T28	202**
		DK427	202**
		Golden Bantam	49 (30, 72)
		Robust	12 (7, 27)
		Seneca Chief	15 (9, 25)
MCPA	<i>Allium cepa</i>	Ishikuro	102 (44, 414)
		Spanish Sweet Utah	152 (59, 504)
		White Pickling	27 (23, 39)
	<i>Lactuca sativa</i>	All Year Round	11 (6, 19)
		Cassandra	51 (n/a)
		Grand Rapids	58 (n/a)
		Tom Thumb	27 (20, 33)
		Triumph	49 (41, 58)
	<i>Raphanus sativus</i>	Cherry Belle	135**
		China Rose	42 (n/a)
		French Breakfast	56 (n/a)
		Minowase	11 (9, 14)
		Sparkler	56 (n/a)
	<i>Solanum lycopersicon</i>	Amish Paste	49 (31, 75)
		Beefsteak	73 (63, 97)
		Long Keeper	39 (24, 63)
		Tiny Tim	65 (56, 95)
		Vendor	69 (47, 88)
	<i>Zea mays</i>	38T28	7590**
		DK427	7590**
		Golden Bantam	3680 (n/a)
		Robust	7590**
		Seneca Chief	310 (140, 899)

Table 2-4. The range in sensitivity to each herbicide tested when using the cultivar with the most and least sensitive IC₅₀ (g ai/ha) for each crop species. Estimated values indicated by an asterisk (refer to Table 2-3).

Herbicide	Crop Species	IC ₅₀ of the Most Sensitive Cultivar	IC ₅₀ of the Least Sensitive Cultivar
Atrazine	<i>Allium cepa</i>	190	1115**
	<i>Lactuca sativa</i>	15	164
	<i>Raphanus sativus</i>	32	227
	<i>Solanum lycopersicon</i>	17	236
Imazethapyr	<i>Allium cepa</i>	1.7	2.3
	<i>Lactuca sativa</i>	208	923**
	<i>Raphanus sativus</i>	16**	16**
	<i>Solanum lycopersicon</i>	9	23**
	<i>Zea mays</i>	12	202**
MCPA Amine	<i>Allium cepa</i>	27	152
	<i>Lactuca sativa</i>	11	58
	<i>Raphanus sativus</i>	11	135**
	<i>Solanum lycopersicon</i>	39	73
	<i>Zea mays</i>	310	7590**

Figure 2-1. Comparison of the effects of the herbicide atrazine on the biomass (expressed as the average percent of the controls) of several cultivars of four crop species.

Figure 2-2. Comparison of the effects of the herbicide imazethapyr on the biomass (expressed as the average percent of the controls) of several cultivars of five crop species.

Figure 2-3. Comparison of the effects of the herbicide MCPA Amine on the biomass (expressed as the average percent of the controls) of several cultivars of five crop species.

Figure 2-4. Range of sensitivity among cultivars for several crop species exposed to three different herbicides. Crops identified by the following: *A. cepa* (A), *L. sativa* (B), *R. sativus* (C), *S. lycopersicon* (D), and *Z. mays* (E).

Figure 2-1.

Figure 2-2.

Figure 2-3.

Figure 2-4.

CHAPTER 3.

Determining the merit of chlorophyll fluorescence and visual rating as endpoints in herbicide toxicity testing.

ABSTRACT

Current pesticide regulatory guidelines, which in part aim to protect non-target vegetation, rely heavily on above-ground plant biomass and a visual rating system when determining herbicide phytotoxicity to both crop and non-crop plant species. Chlorophyll fluorescence has been used for detecting early, non-visual herbicidal effects on various plant species, but its reliability as an endpoint in risk assessment is unknown. The validity of visual rating and chlorophyll fluorescence as reliable endpoints in ecological risk assessment was investigated during this study. Six different plant species (three crops and three closely related wild relatives) were exposed to the commercial herbicides atrazine and glyphosate in two separate experiments which included five herbicide doses plus a control. Chlorophyll fluorescence measurements were taken and visual observations were recorded on 0, 1, 7, 17, and 28 days after herbicide exposure. Above-ground plant biomass was collected and a visual rating was assigned at 28 days after herbicide application (the final day of testing). The concentration resulting in a 20% inhibition compared to controls (IC_{20}) was determined for each species and herbicide for the endpoints biomass, visual rating, and chlorophyll fluorescence. It was found that chlorophyll fluorescence is an excellent tool for the early detection of herbicidal effects, and that both chlorophyll fluorescence and visual rating can be used to aid in the prediction of herbicide phytotoxicity and provide additional information to biomass data

when performing risk assessments. However, chlorophyll fluorescence and visual rating should not be relied on exclusively for prediction of inhibition concentrations in herbicide regulatory testing. When compared to biomass, fluorescence and visual rating almost always under-estimated the phytotoxicity of both atrazine and glyphosate.

INTRODUCTION

It is well documented that the drift of agricultural herbicides poses a threat to non-target vegetation in arable field margins and nearby woodlots and hedgerows (Marrs et al., 1989; Marrs et al., 1991; Kleijn and Snoeijs, 1997; de Snoo and van der Poll, 1999; Follak and Hurle, 2003). Damage caused by herbicide exposure can produce changes in the development, growth, or morphology of sensitive plant species (Freemark and Boutin, 1995) and has also been shown to affect plants at the community level leading to changes in abundance, diversity, and composition of species in both crop and non-crop habitats (Marshall and Bernie, 1985; de Snoo, 1997; Kleijn and Snoeijs, 1997; Boutin and Jobin, 1998; de Snoo, 1999; Boutin et al., 2002).

Since 1931, when Kautsky and Hirsch first identified chlorophyll fluorescence, it has increased in popularity as a tool for assessing the photosynthetic performance and general physiological state of plants when exposed to various types of stresses such as suboptimal light, extreme temperatures, drought, and environmental pollutants (e.g. herbicides, heavy metals, air pollutants, etc.) (Smillie and Hetherington, 1983; Krugh and Miles, 1996; Juneau and Popovic, 1999; Franzaring et al., 2001; Korres et al., 2003; Marwood et al., 2003).

The principle underlying chlorophyll fluorescence is relatively simple. Light energy is absorbed by the antenna pigments in the photosynthetic apparatus found in the chlorophyll molecules of plant leaves (Krause and Weis, 1991) and the resulting supply of excitation energy is transferred to the reaction centres of the two photosystems where approximately 85% is used in photosynthesis (Papageorgiou, 1975). The remaining portion of energy is dissipated mainly as heat or re-emitted as light in the form of chlorophyll fluorescence (Schreiber, 1983; Govindjee, 1995). Although the production of chlorophyll fluorescence constitutes only approximately 1-2% of the total light energy, its spectrum is different than that of the absorbed light so measurements are easily done with the proper instrumentation (Maxwell and Johnson, 2000). Major advantages of chlorophyll fluorescence are that it is non-destructive and non-invasive, as well as rapid to measure and highly sensitive (Bolhàr-Nordenkamp et al., 1989; Schreiber et al., 1998; Lazár, 1999).

Chlorophyll fluorescence can provide extensive information on the intactness of photosystem II (PS II), thylakoid membrane degradation, electron transport efficiency, and overall photosynthetic function, not to mention other physiological processes that are indirectly affecting the normal functioning of the photosynthetic apparatus (Govindjee, 1995; Rosenqvist and van Kooten, 2003).

To date, non-target plant testing for ecological risk assessment has relied heavily on visual rating and above-ground biomass of crop species for the determination of herbicide phytotoxicity (Holst and Ellwanger, 1982; OECD, 1984; OECD, 2005). Although these endpoints are commonly used for the determination of plant herbicide sensitivity, the results are rarely taken into account simultaneously and both have their

individual weaknesses. Visual rating data can be quite subjective and therefore are not necessarily accurate; above-ground plant biomass may not reflect herbicidal effects that alter the overall health and physiology of the plant in other ways aside from a reduction in biomass. Since chlorophyll fluorescence has been shown to be both rapid and effective at detecting herbicidal effects on various plant species (Conrad et al., 1993; Snel et al., 1998; Matoušková et al., 1999; Dewez et al., 2002) it may be a useful endpoint to include in current pesticide regulatory guidelines to aid in the determination of herbicide phytotoxicity.

The objectives of this study were: 1) to determine whether chlorophyll fluorescence is a useful predictor of herbicidal effects on both crop and non-crop plant species; 2) to investigate whether chlorophyll fluorescence and/or visual rating are complementary to plant biomass for the determination of herbicide phytotoxicity in regulatory testing.

MATERIALS AND METHODS

This experiment was conducted in the greenhouses at the National Wildlife Research Centre and the Nesbitt Biology building located at Carleton University in Ottawa Ontario, between March 2004 and September 2005.

Herbicides Selected

Two commercial agricultural herbicides, Aatrex (480 g ai/L) and Round-Up Original (356 g ai/L), containing atrazine and glyphosate, respectively, were selected for testing. The herbicides will be referred to by their common names, atrazine and

glyphosate, throughout this study. These particular herbicides were chosen because both are commonly used throughout Canada for agricultural, industrial, and domestic purposes, as well as represent different classes of herbicides with different modes of action (OMAFRA, 2002). Atrazine is a triazine herbicide that inhibits photosynthesis, while glyphosate inhibits amino acid synthesis (Thomson, 2002). Detailed descriptions of each herbicide can be found in Appendix 1.

Plant Species Selected and Requirements for Growth

Three crop species, strawberry (*Fragaria ananassa*), lettuce (*Lactuca sativa*), and tomato (*Solanum lycopersicon*), were chosen for this study. The closest non-crop, native relative to each of the crop species was also selected for testing, which included thick-leaved wild strawberry (*Fragaria virginiana*), tall lettuce (*Lactuca canadensis*), and black nightshade (*Solanum nigrum*). Three different families are represented by the six plant species selected for this experiment: Rosaceae (*Fragaria spp.*), Solanaceae (*Solanum spp.*), and Asteraceae (*Lactuca spp.*).

All crop species were germinated in moist vermiculite and transplanted into 10 cm plastic pots containing a rich potting mixture within seven days of germination. All crop species germinated within seven days of planting. *L. sativa* and *S. lycopersicon* had germination rates of 80-90%. *F. ananassa* had a relatively lower germination with a rate of 60-70%. Non-crop species, although varied in their germination requirements, all had the same germination rate of 75-80%. Detailed germination requirements of non-crop species can be found in Appendix 2.

Experimental Treatments and Protocol

Initially, 21-day screening tests using five doses (three replicates per dose) ranging from 0-100% of the herbicide label rate were performed to determine the sensitivity range of each species to both atrazine and glyphosate. Herbicides were applied using a track spray booth equipped with a TeeJet 8002E flat-fan nozzle, delivering 6.75 ml/m² at 206.84 kPa (de Vries Manufacturing). Chosen label rates for atrazine and glyphosate were 1296 g ai/ha and 2136 g ai/ha, respectively.

After screening tests were complete and the herbicide sensitivity range of each species was determined, a definitive test for each species was performed. Separate experiments were conducted with atrazine and glyphosate for each of the species chosen. Six doses (including controls), and five replicates per dose were included for both atrazine and glyphosate. Definitive test doses were chosen to span the sensitivity range determined in the screening test and followed a geometric progression from lowest to highest dose. The definitive doses applied to each species can be found in Appendix 3. All plant species were exposed to a one-time application of the herbicides at the 3-6 leaf stage. Plants were randomly assigned a numerical identification tag to prevent bias during measurements.

Visual observations were noted for every plant just before herbicide application, and at 1, 7, 17, and 28 days after application. At 28 days after herbicide exposure, all aboveground green plant material was harvested and placed in a forced-air dryer for 72 hours at approximately 70°C for biomass determination. Also at day 28, a visual rating ranging from 0 to 9 was given to each plant, where each score represented a degree of herbicidal effect based on visual observations (Table 3-1).

Fluorescence Analyses

Chlorophyll fluorescence measurements were recorded under steady-state conditions for three plants per herbicide dose, along with three controls, for both atrazine and glyphosate using a kinetic fluorescence CCD camera (Photon Systems Instruments, Czech Republic). Plants were subjected to an actinic light with a wavelength of 625 nm and an intensity of 300 μE ; saturating light pulses were 1600 ms in duration and had an intensity of 4000 μE . Plants were dark-adapted for 30 minutes (an assumption of the Kautsky curve) prior to the collection of fluorescence measurements. Chlorophyll fluorescence measurements were taken on the entire plant to coincide with the visual observations gathered at days 0, 1, 7, 17, and 28 after herbicide application.

The maximum quantum yield and achievable efficiency of PSII, represented by F_v/F_m , was calculated based on the Kautsky curve produced by each plant. F_v/F_m is given by the equation: $F_v/F_m = (F_m - F_o) / F_m$ (where F_m represents the maximum fluorescence and F_o represents the minimal fluorescence in dark-adapted plant tissue) and usually ranges between 0.75 and 0.85 for healthy plant tissue (Bolhr-Nordenkampf et al., 1989). The ratio F_v/F_m is one of the most commonly used chlorophyll fluorescence parameters for detecting plant stress. Also calculated from the Kautsky curve, was a vitality index (Rfd) that indicates the potential overall photosynthetic function including activity of the Calvin cycle and related processes (Lichtenthaler and Rinderle, 1988). Rfd is given by the equation: $\text{Rfd} = (F_m - F_t) / F_m$ (where F_m represents the maximum fluorescence and F_t represents the steady-state fluorescence level). High Rfd values (usually greater than 2.5) indicate good photosynthetic activity, however as leaf age increases, Rfd values

decline (Lichtenthaler and Rinderle, 1988). Rfd values below 1.0 indicate a severe disturbance to overall photosynthetic function.

Data Analyses

For each species and herbicide, a one-way analysis of variance (ANOVA) was performed for days 0, 1, 7, 17, and 28 after herbicide application to determine when significant differences between doses for the fluorescence parameters F_v/F_m and Rfd compared to controls occurred. In several situations, the assumptions of the ANOVA were not met (even after transformations) so the non-parametric Kruskal-Wallis test was performed.

The following endpoints: visual rating (day 28), and F_v/F_m and Rfd (days 7 and 17), were compared to biomass at day 28 using bivariate correlation for each species and herbicide. Spearman's rho and significance values were recorded and used to determine which of these endpoints showed the strongest correlation to biomass. Biomass was chosen as the endpoint to compare to other measurements because it is most commonly used in regulatory testing and is not a subjective integrated measure like visual assessment.

An IC_{20} (defined as the effective dosage that results in a 20% reduction compared to the controls) was calculated for every species and herbicide using each of the parameters biomass (day 28), visual rating (day 28), and F_v/F_m (day seven). All IC_{20} were calculated using the linear interpolation method for sub lethal toxicity, also known as the inhibition concentration approach (IC_p) as described in the US EPA report EPA/600/4/-89-001 and 001A.

EXPERIMENTAL RESULTS

Analysis of fluorescence measurements showed that for both atrazine and glyphosate, F_v/F_m and Rfd are both effective parameters for providing early detection of herbicidal effects on both crop and non-crop plant species (Figures 3-1 to 3-4). Days in which a significant ($p < 0.05$) reduction in fluorescence was detected are indicated on the figures by an asterisk.

F_v/F_m was significantly reduced one day after exposure to atrazine for all plants except for *L. canadensis* and *S. nigrum* (Figure 3-1). However, significant differences between doses of atrazine occurred for all species by day seven. The effects of glyphosate on F_v/F_m for the species tested were not as obvious and in general did not occur as quickly as they did for atrazine (Figure 3-2). *S. lycopersicon* and *S. nigrum* were the only species to exhibit significant differences between doses one day after herbicide exposure. Both *Lactuca* species tested showed significant differences in F_v/F_m by seven days after glyphosate application. Due to poor dose-response, there were no significant differences between doses of glyphosate at any time for *F. ananassa*; *F. virginiana* did not exhibit significant differences until 28 days after exposure to glyphosate.

A significant reduction in Rfd was evident one day after exposure to atrazine for half the species tested (*L. canadensis* and both *Solanum* species) (Figure 3-3). However, all species showed obvious reductions in Rfd values one day after spray, but within-species variability may have resulted in statistical non-significance. *F. virginiana*, *L. sativa*, and *S. nigrum* all showed significant differences between doses for Rfd as early as one day after exposure to glyphosate (Figure 3-4). *S. lycopersicon* and *L. canadensis* were slower to respond but Rfd values were significantly different between doses by

seven days after glyphosate application. *F. ananassa* did not show statistically significant differences between doses for Rfd at any time during the test period.

Rfd is a more variable parameter than F_v/F_m , with significant differences detected in two occasions before herbicide application among plants that appeared equally healthy, and for many species, both F_v/F_m and Rfd showed some level of recovery back to control levels by the end of the test period.

Regardless of when herbicidal effects were detected using chlorophyll fluorescence, visual effects were not obvious until a minimum of seven days after exposure for all species and herbicides. In general, plants exposed to atrazine exhibited mottled leaves and eventually some degree of withering; plants exposed to glyphosate also became withered depending on the level of response, along with young leaf and bud malformations.

For each species and herbicide, the visual rating at day 28, and F_v/F_m and Rfd (days 7 and 17 after herbicide exposure) were compared to biomass at 28 days after herbicide exposure. F_v/F_m and Rfd were selected at day 7 and 17 since this is when significant effects were most pronounced. The Spearman's rho and significance values for each correlation are shown in Table 3-2. Untreated controls were removed from these correlations.

For plants sprayed with atrazine, F_v/F_m at day 17 showed the strongest relationship with biomass, with significant correlations for every species (Table 3-2). However, F_v/F_m at day seven and visual rating at day 28 were also strongly correlated to biomass, with *L. canadensis* being the only species exhibiting a slightly insignificant correlation for both parameters. Rfd showed a relatively strong relationship with

biomass at day 7 and slightly less so at day 17 after herbicide exposure. Plants sprayed with glyphosate did not exhibit as strong a correlation between biomass and F_v/F_m compared to those exposed to atrazine, but visual rating and biomass were closely related for all species tested. Rfd (at 7 and 17 days after spray) did not show as close a relationship with biomass as that found with plants sprayed with atrazine.

Based on the values shown in Table 3-2 and the results presented in Figures 3-1 and 3-2, F_v/F_m (at day seven), and visual rating and biomass (both at day 28), were selected for further investigation regarding the comparability of these endpoints in predicting phytotoxicity. F_v/F_m (at day 17) was not selected for IC_{20} calculation, because although there are strong correlations with biomass, recovery had begun to occur for many species and therefore is not as good (responses < 20%) as day seven where effects are the most prominent for most species. Rfd was not investigated further because this parameter did not show a consistently strong correlation to biomass for both herbicides tested compared to F_v/F_m . The IC_{20} for each species and herbicide, using each selected endpoint, calculated using the linear interpolation method is shown in Table 3-3.

For atrazine, F_v/F_m is a slightly better predictor of herbicide phytotoxicity than visual rating, producing similar IC_{20} at seven days after herbicide application as biomass at 28 days after spray. However, both F_v/F_m and visual rating predicted a slightly higher IC_{20} in comparison to biomass for all species. An IC_{20} could not be calculated for *F. ananassa* using visual rating because no herbicidal effects could be observed.

IC_{20} calculated using visual rating and biomass were more similar compared to those determined using F_v/F_m for plants sprayed with glyphosate. IC_{20} could not be

calculated for either *Fragaria* species using F_v/F_m at day seven due to the lack of effects detected by this parameter.

DISCUSSION

Current pesticide regulatory guidelines rely heavily on above-ground plant biomass and visual rating when determining herbicide phytotoxicity to both crop and non-crop plant species. Using visual rating as an endpoint in regulatory testing is somewhat controversial since this method is a subjective measurement based solely on visual observations. The validity of visual rating as a reliable endpoint was investigated during this study, along with whether the technique of chlorophyll fluorescence is an effective predictor of herbicidal effects and a useful endpoint in regulatory testing.

The results of this experiment show that chlorophyll fluorescence is an excellent tool for the early prediction of effects caused by atrazine and glyphosate exposure to both crop and non-crop species. Using chlorophyll fluorescence, herbicidal effects that resulted in significant reductions in PSII efficiency (F_v/F_m) were detected as early as one day after spray for plants exposed to atrazine; plants exposed to glyphosate showed significant reductions at one day, but mostly by seven days, after herbicide application. Overall photosynthetic function was also monitored using Rfd, but in general was not significantly affected until seven days after spray, with the exception of a few plant species.

Chlorophyll fluorescence has previously been shown to be a useful tool in assessing photosynthetic performance and overall plant health after herbicide exposure (Matoušková et al., 1999; Franzaring et al., 2001; Korres et al., 2003). However, the

previously mentioned experiments included fewer herbicide concentrations, had shorter test periods, and for the exception of Franzaring et al. (2001) did not include non-crop plant species. Another important difference to note was that in this study chlorophyll fluorescence measurements were an integrated measure of the entire plant and collected throughout the 28 day test period, whereas in the other studies measurements were taken only within the first 48 hour period after herbicide exposure, and on single leaves only.

Chlorophyll fluorescence measurements are not only useful for the prediction of early herbicidal effects on specific plants and/or species; they may also provide insight into the potential long-term ecological consequences resulting from herbicide exposure. In this experiment, it was shown that chlorophyll fluorescence has the ability to detect herbicidal stress prior to the appearance of visual effects, which agrees with the findings of Franzaring et al. (2001). It was also found in this study, similar to the data reported by Matoušková et al. (1999), and Korres et al. (2003), that some plant species are able to survive, albeit with reduced biomass, low doses of the herbicides included in this experiment. However, based on the findings of this study, and consistent with those of Kleijn and Snoeijing (1997), the severity of herbicidal effects may have different consequences on plants with different lifecycles. Annual species, such as *S. nigrum* included in this study, may not have time to recover and reproduce from the lag period induced by herbicide exposure. Alternatively, perennial species, like *F. virginiana*, may reproduce in subsequent years but a reduction in biomass may influence their ability to compete successfully with other species. Also, Franzaring et al. (2001) showed that the extent of foliar injury suffered by the plant species included in their study resulting from exposure to ethofumesate vapours (whose mode of action inhibit fatty acid synthesis,

cuticle development, and meristematic growth) was related to the relative growth rate as well as the specific leaf area associated with individual species. Marrs et al. (1989) suggested that populations of a sensitive species could decline over time due to the inability to compete with less affected species. Long-term experiments that investigate interactions are necessary to determine the subtle effects that may be associated with sub-lethal herbicide exposure throughout the duration of a plant's lifecycle, as well as between-plant interactions.

Chlorophyll fluorescence parameters (seven days after herbicide exposure) and visual rating (28 days after herbicide exposure) were compared to plant biomass on day 28 to determine which of these parameters were most closely linked to biomass. For species exposed to atrazine the chlorophyll fluorescence parameter F_v/F_m was slightly more often correlated with biomass than visual rating, while visual rating was more strongly linked to biomass than the fluorescence for plants sprayed with glyphosate. Christensen et al. (2003) found similar results, but the aim of their study was slightly different in that fluorescence was being investigated for the purpose of rapid identification of herbicidal effects for use in herbicide screening tests within the first 48 hours after herbicide application. However, the authors found that the best predictor of plant biomass using fluorescence was for plants exposed to the PSII inhibitor bentazon. Plants sprayed with glyphosate and fluorochloridone (a phytoene desaturase inhibitor) did not exhibit as strong relationships with biomass and the fluorescence parameters tested in their experiment.

The results found in this study, as well as that performed by Christensen et al. (2003) may be related to the differing mode of action of the herbicides tested. It is not

surprising that PSII inhibiting herbicides (e.g. atrazine and bentazon) would show a stronger relationship between chlorophyll fluorescence and biomass than herbicides that do not directly affect the photosynthetic apparatus. However, Abbaspoor and Streibig (2005) reported that a strong link between biomass and several fluorescence parameters existed for plants exposed to clodinafop (an acetyl-coenzyme A carboxylase inhibitor).

Visual rating was found to be a reliable endpoint in herbicide toxicity prediction compared to biomass for plants sprayed with glyphosate and only slightly less effective for those sprayed with atrazine.

Based on the results gathered in this experiment, chlorophyll fluorescence and visual rating were found to be closely linked to plant biomass depending on the herbicide tested. For atrazine, chlorophyll fluorescence, visual rating, and biomass produced similar inhibition concentrations for most species. Visual rating and biomass gave comparable inhibition concentrations for plants exposed to glyphosate, but chlorophyll fluorescence was not as effective. Knowing this, these parameters could be used to aid in the prediction of herbicide phytotoxicity and provide additional information when performing risk assessments. However, chlorophyll fluorescence and visual rating should not be relied on exclusively for prediction of inhibition concentrations in herbicide regulatory testing. When compared to biomass, fluorescence and visual rating almost always under-estimated the phytotoxicity of both atrazine and glyphosate. Christensen et al. (2003) also found that the fluorescence parameters tested in their experiment often predicted higher ED₅₀ (doses resulting in a response 50% of the controls) compared to plant biomass for the herbicides tested.

CONCLUSION

Chlorophyll fluorescence is an excellent predictor of early, non-visual herbicidal effects for both atrazine and glyphosate. In addition, chlorophyll fluorescence and visual rating can effectively predict inhibition comparable to plant biomass depending on the herbicide tested. However, since both of these parameters under-estimated the phytotoxicity of atrazine and glyphosate, plant biomass (dry weight) should remain the preferred endpoint when determining herbicide phytotoxicity in regulatory testing.

Table 3-1. Visual rating scores corresponding to plant growth and vigour after herbicide exposure (adapted from Hamill et al., 1977).

Visual Rating	Range of Inhibition (%)	Midpoint (%)	Corresponding Visual Observations
0	0	0	No effect
1	1 – 5	3	Trace effect; hormesis
2	6 – 15	10.5	Slight effect
3	16 – 29	22.5	Moderate effect; plants 75% of controls
4	30 – 44	37	Plants > 50% of controls but have some visible injury
5	45 – 64	54.5	Plants ~ 50% of controls with definite injury
6	65 – 79	72	Plants ~ 25% of controls with definite injury
7	80 – 90	85	Plants < 25% of controls with definite injury
8	91 – 99	95	Approaching complete kill; little green tissue remaining
9	100	100	Complete kill; no green tissue

Table 3-2. Spearman's rho and significance values (shown in brackets) for correlations between plant biomass at 28 days after herbicide application and other potential endpoints (ie. visual rating and chlorophyll fluorescence).

Herbicide	Species	Visual Rating	Fv/Fm Day 7	Fv/Fm Day 17	Rfd Day 7	Rfd Day 17
Atrazine	<i>F. ananassa</i>	0.503 (0.005)	0.624 (0.013)	0.648 (0.009)	0.1 (0.723)	0.036 (0.899)
	<i>F. virginiana</i>	0.917 (< 0.001)	0.793 (< 0.001)	0.793 (< 0.001)	0.825 (< 0.001)	0.811 (< 0.001)
	<i>L. sativa</i>	0.984 (< 0.001)	0.791 (< 0.001)	0.99 (< 0.001)	0.785 (< 0.001)	0.99 (< 0.001)
	<i>L. canadensis</i>	0.395 (0.078)	0.452 (0.091)	0.579 (0.024)	0.626 (0.013)	0.558 (0.031)
	<i>S. lycopersicon</i>	0.967 (< 0.001)	0.929 (< 0.001)	0.768 (0.001)	0.929 (< 0.001)	0.821 (< 0.001)
	<i>S. nigrum</i>	0.967 (< 0.001)	0.874 (< 0.001)	0.613 (0.015)	0.67 (0.006)	0.258 (0.354)
Glyphosate	<i>F. ananassa</i>	0.721 (< 0.001)	0.502 (0.068)	0.079 (0.788)	-0.243 (0.402)	-0.293 (0.310)
	<i>F. virginiana</i>	0.765 (< 0.001)	0.186 (0.507)	0.513 (0.051)	0.061 (0.83)	0.311 (0.2)
	<i>L. sativa</i>	0.972 (< 0.001)	0.642 (0.01)	0.895 (< 0.001)	0.617 (0.014)	0.904 (< 0.001)
	<i>L. canadensis</i>	0.877 (< 0.001)	0.961 (< 0.001)	0.953 (< 0.001)	0.825 (< 0.001)	0.296 (0.283)
	<i>S. lycopersicon</i>	0.805 (< 0.001)	0.8 (< 0.001)	0.639 (0.01)	0.886 (< 0.001)	0.616 (0.014)
	<i>S. nigrum</i>	0.972 (< 0.001)	0.861 (< 0.001)	0.746 (0.001)	0.854 (< 0.001)	0.855 (< 0.001)

Table 3-3. Inhibition concentrations (IC_{20}) for each species and herbicide calculated using the interpolation method based on plant biomass, visual assessment, and a selected chlorophyll fluorescence parameter (F_v/F_m). Confidence intervals are shown in brackets.

Herbicide	Species	Lowest Dose Tested (g ai/ha)	Highest Dose Tested (g ai/ha)	Biomass Day 28	F_v/F_m Day 7	Visual Rating Day 28
Atrazine	<i>F. ananassa</i>	26	130	79 (24, 104)	61 (52, 79)	n/a
	<i>F. virginiana</i>	26	130	13 (9, 25)	43 (36, 70)	36 (21, 43)
	<i>L. sativa</i>	104	259	1.99 (1.7, 2.5)	9 (6, 16)	5 (2, 104)
	<i>L. canadensis</i>	13	130	82 (n/a)	86 (58, 117)	111 (n/a)
	<i>S. lycopersicon</i>	130	376	44 (41, 47)	51 (42, 73)	115 (75, 171)
	<i>S. nigrum</i>	130	570	7 (4, 63)	27 (18, 83)	17 (8, 127)
Glyphosate	<i>F. ananassa</i>	43	171	27 (19, 72)	n/a	30 (21, 71)
	<i>F. virginiana</i>	214	1089	3.1 (2.8, 3.6)	n/a	4 (3.6, 4)
	<i>L. sativa</i>	320	662	1.96 (1.7, 2.4)	8 (7, 16)	5 (3, 104)
	<i>L. canadensis</i>	43	983	100 (64, 138)	262 (180, 559)	85 (68, 127)
	<i>S. lycopersicon</i>	21	171	46 (19, 61)	159 (151, 166)	41 (34, 58)
	<i>S. nigrum</i>	171	876	5 (4, 12)	633 (599, 675)	6 (4, 10)

Figure 3-1. The effect of atrazine on the chlorophyll fluorescence parameter F_v/F_m at 0, 1, 7, 17, and 28 days after herbicide application. Days in which doses are significantly different from each other are indicated by an asterisk.

Figure 3-2. The effect of glyphosate on the chlorophyll fluorescence parameter F_v/F_m at 0, 1, 7, 17, and 28 days after herbicide application. Days in which doses are significantly different from each other are indicated by an asterisk.

Figure 3-3. The effect of atrazine on the chlorophyll fluorescence parameter R_{fd} at 0, 1, 7, 17, and 28 days after herbicide application. Days in which doses are significantly different from each other are indicated by an asterisk.

Figure 3-4. The effect of glyphosate on the chlorophyll fluorescence parameter R_{fd} at 0, 1, 7, 17, and 28 days after herbicide application. Days in which doses are significantly different from each other are indicated by an asterisk.

Figure 3-1.

Figure 3-2.

Figure 3-3.

Figure 3-4.

CHAPTER 4.

Comparing the toxicity of the herbicide Round-Up Original[®] and its active ingredient glyphosate to eight different terrestrial plant species.

ABSTRACT

Glyphosate is found in many different commercial herbicides and is one of the most commonly applied herbicidal active ingredients in the world. Although pesticide formulations may contain additional ingredients that may result in an increase in overall toxicity, North American pesticide regulatory guidelines require toxicity testing exclusively of the active ingredient and not the pesticide formulation or tank mixes. In this study, comparison of the phytotoxicity of the tank-mix formulation of Round-Up Original[®] to its active ingredient glyphosate on eight different terrestrial plant species was investigated. Results showed that glyphosate active ingredient was much less toxic than its formulated product for seven of eight species tested. The data suggest that regulatory guidelines should consider the formulated products, along with any surfactants or adjuvants that may be added if these chemicals are suggested on the herbicide label, when evaluating pesticide toxicity.

INTRODUCTION

Glyphosate [(N-phosphonomethyl) glycine] is one of the most widely used global herbicides due to its excellent efficacy to control of annuals, perennials, and woody species (Baylis, 2000). In Ontario, Canada, total glyphosate usage for agricultural purposes has increased 58% since 1998 (McGee et al., 2004), which could likely be

attributed to the increasing use of genetically modified crops exhibiting glyphosate resistance. In plant species, glyphosate inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), which results in an accumulation of shikimate-3-phosphate, eventually preventing aromatic amino acid production, and thus inhibiting protein synthesis; other physiological and chemical effects have also been noted (Baylis, 2000).

Pesticide regulatory guidelines in Canada and in the United States currently require toxicity testing of the active ingredient and not the pesticide formulation (Holst and Ellwanger, 1982). Every formulated pesticide contains many other chemicals besides the active ingredient. Although considered inert, these additives can result in a greater pesticide efficacy. In general, inert ingredients are not listed on pesticide labels because this information is proprietary.

Studies have shown that glyphosate formulations are often more toxic than the active ingredient to different species of invertebrates and fish (Folmar et al., 1979), frogs (Mann and Bidwell, 1999; Howe et al., 2004), and algae (Sáenz et al., 1997). There has also been extensive research regarding the toxicity of different glyphosate formulation additives. It has been reported that these additives, such as surfactants and adjuvants, are quite effective at increasing the toxicity of active ingredients to plant species (Wyrill and Burnside, 1977; Buhler and Burnside, 1983; Nalewaja and Matysiak, 1992; Riechers et al., 1995; Sharma and Singh, 2001). Formulation additives allow improved absorption and translocation of the chemical active (Gaskin and Holloway, 1992; Nalewaja et al., 1996; Sharma and Singh, 2000; Molin and Hirase, 2004), and can result in differences in toxicity between formulations (Fairchild et al., 2002).

The objectives of this study were to: 1) determine if differences in phytotoxicity exist between glyphosate and the tank-mix of formulated product Round-Up Original[®] to both crop species and non-crop plant species, and 2) what implications, if any, the results may have on current pesticide registration guidelines.

MATERIALS AND METHODS

This experiment was conducted in the greenhouses at the National Wildlife Research Centre located at Carleton University in Ottawa, Ontario, between December 2004 and September 2005.

Herbicide Selected

The commercial formulated herbicide Round-Up Original[®], which contains the active ingredient glyphosate [N-(phosphonomethyl) glycine] in the form of isopropylamine salt, was selected for this study. The formulated product, Round-Up Original[®], was obtained from a commercial retail outlet, and the active ingredient glyphosate was obtained from Monsanto Canada, Inc. Glyphosate is a non-selective systemic herbicide used for the control of annual and perennial grasses and broad-leaved plant species, as well as woody brush and trees, in agricultural, industrial, and domestic situations (Monsanto Canada Inc., 2001; Tomlin, 2003). This herbicide is also commonly used on resistant genetically modified crops such as soybeans and canola.

Plant Species Selection and Germination Requirements

Four common crop species were included in this experiment: soybean (*Glycine max*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicon*), and corn (*Zea mays*). Four wild plant species were also selected for inclusion in this study: big bluestem (*Andropogon gerardii*), tall lettuce (*Lactuca canadensis*), black nightshade (*Solanum nigrum*), and American vetch (*Vicia americana*). The eight plant species selected for this experiment represent four different families: Asteraceae, Fabaceae, Solanaceae, and Poaceae.

All species were germinated in moist vermiculite and transplanted into 10 cm plastic pots containing a rich potting mixture within seven days of germination. All crop species germinated within seven days of planting and had germination rates of 80-95%. Non-crop species, although varied in their germination requirements (see Appendix 2), had germination rates between 75 – 95%.

Experimental Treatments and Protocol

Initially, 21-day screening tests using five doses (three replicates per dose) ranging from 0 – 100% of the herbicide label rate were performed to determine the sensitivity range of each species to Round-Up Original[®]. A non-ionic surfactant (Agral 90[®]) containing the chemical nonylphenoxy polyethoxyethanol was added to the formulated product as the herbicide label indicated at a rate of 350 ml/ha for 50-100 L water. The herbicide was applied using a track spray booth equipped with a TeeJet 8002E flat-fan nozzle, delivering 6.75 ml/m³ at 206.84 kPa (de Vries Manufacturing).

After screening tests were complete, a definitive test was performed. Separate experiments were conducted with the formulated product Round-Up Original[®] and the active ingredient glyphosate. Plants were randomly assigned a numerical identification tag to prevent bias during measurements. The surfactant was added to both the active ingredient and the formulated product at the same rate as the screening test, and both treatments utilized clean tap water as the solvent. Definitive test doses were chosen to span the sensitivity range determined in the screening test and followed a geometric progression from lowest to highest dose (see Appendix 2). For the definitive test, six doses (including controls) and five replicates per dose were included for both Round-Up Original[®] and glyphosate. For *S. nigrum*, only three replicates per dose were included for the definitive test containing glyphosate due to insect infestation.

Before herbicide exposure, and at 28 days after spray, visual observations were recorded for each species and treatment. Also at 28 days after herbicide exposure, all aboveground green plant material was harvested for each sample and placed in a forced-air dryer for 72 hours at approximately 70°C for biomass determination.

Data Analyses

For each species and treatment (Round-Up Original[®] or glyphosate) an IC₂₅ and an IC₅₀ (defined as the effective dose that results in a 25% and 50% reduction in biomass compared to the controls, respectively) was calculated using the linear interpolation method for sub lethal toxicity, also known as the inhibition concentration approach (IC_p) as described in the US EPA report EPA/600/4/-89-001 and 001A.

For each species, a two-way analysis of variance (ANOVA) was performed to compare differences between the two herbicide treatments, their dose-responses, and any interactions for each species. All assumptions for an ANOVA were met for all species. The average proportion of the controls (shown as a percent) were used rather than the biomass to eliminate slight differences that sometimes arose between individuals of the same species that were grown at different times in the greenhouse. In most cases, however, definitive tests were performed at the same time for Round-Up Original[®] and glyphosate.

EXPERIMENTAL RESULTS

Table 4-1 shows the IC₂₅ and IC₅₀ for each species and treatment. In six out of eight species the IC₅₀ could not be calculated due to lack of 50% effect. The IC₂₅ and the IC₅₀ could not be calculated for either treatment for *A. gerardii* due to erratic response of this species. However, comparable dose-response was observed for this species for both Round-Up Original[®] and glyphosate. Neither IC₂₅ nor IC₅₀ could be calculated for *Z. mays* exposed to glyphosate due to poor dose-response; values were calculated for Round-Up Original[®]. The dose-response curves for every species tested comparing the biomass (expressed as a percent of the controls) between plants exposed to Round-Up Original[®] or glyphosate are shown in Figure 4-1. Some level of visual effects such as leaf mottling, withering, and malformations of buds and younger leaves were noted for every species depending on the intensity of response to either treatment. Visual effects were similar between both treatments and only differed in the extent of damage caused.

For all species, except *S. nigrum* and *A. gerardii*, the IC_{25} was much lower for the formulated product than it was for the active ingredient alone, indicating that glyphosate is much less toxic to the species tested than the formulated product Round-Up Original[®]. The opposite response exhibited by *S. nigrum* may not necessarily represent true results since this species was unhealthy during the test period due to heavy insect infestation for both herbicide treatments as well as controls. An IC_{50} could not be calculated for six out of eight species exposed to glyphosate; an IC_{50} was determined for all species exposed to Round-Up except for *A. gerardii*.

The two-way ANOVAs performed for each species comparing the effects of treatment and dose on plant biomass indicated that significant differences in response existed between Round-Up Original[®] and glyphosate for every species (Table 4-2). Dose significantly affects biomass. For all but three species (*A. gerardii*, *L. canadensis*, and *L. sativa*), significant interactions were found between dose and treatment which indicates the way in which dose affects biomass differs between Round-Up Original[®] and glyphosate for some plants.

DISCUSSION

Differences in toxicity between glyphosate and combinations of glyphosate with different surfactants to control various species of plants are known (Wyrill and Burnside, 1977; Riechers et al., 1995; Sharma and Singh, 2001; Fairchild et al., 2002). This experiment differed from the abovementioned studies in that any increased toxicity due to surfactant was controlled for by adding the surfactant Agral-90[®] in a rate recommended on the label of Round-Up Original[®] to both the active ingredient and the formulated

product. In doing this, the phytotoxicity of the tank-mix applied in field situations could be determined, and the toxicity of any unknown ingredients (often considered inert) could be revealed.

This study included two treatments: the complete tank-mix formulation (active ingredient + unknown inert ingredients + pre-spray surfactant) that would be actually sprayed onto an agricultural field in an attempt to control weeds, and the most likely chemical combination submitted for risk assessment by registrants for regulatory purposes (active ingredient + surfactant). Three additional treatments (formulated product + no surfactant, active ingredient + no surfactant, and surfactant only) could have been included in this experiment to investigate the toxicity of Agral-90[®]. However, this study did not endeavor to determine the toxicity of the surfactant, nor did it attempt to explain why or how formulated products may be more toxic than the active ingredient. It was simply designed to determine if the tank-mix of Round-Up Original[®] is more toxic than the combination of active ingredient plus surfactant. Any variation in phytotoxicity between the two chemical treatments is likely due to the unknown “inerts” contained in the formulated product.

Significant differences in phytotoxicity were found between the formulated product Round-Up Original[®] and its active ingredient glyphosate. A similar study by Molin and Hirase (2004) reported significant difference in phytotoxicity between three different formulated herbicides containing glyphosate. These results also indicate that the unknown “inert” ingredients in those herbicides affect glyphosate efficacy. Using current regulatory protocol, the toxicity of the formulation Round-Up Original[®] plus the added surfactant Agral-90[®], in regards to plant species, would be highly underestimated.

Since regulatory agencies only test the active ingredient, the estimated phytotoxicity would likely be even lower since the surfactant may not have been included. How additives will affect the herbicide efficacy can be difficult to predict and quite variable (Wyrill and Burnside, 1977). Knowing this, the complete tank-mix to be sprayed in the field should be included in phytotoxicity testing to allow for the toxicity of the unknown ingredients contained in the formulated product, along with the surfactant that may be added prior to spray.

Many more active ingredients must be compared to their formulated products before broad conclusions can be made, however, this type of experiment would be difficult to execute since it is a challenge to obtain the active ingredients in the technical active form. Nonetheless, the results gathered in this study, along with previous research, strongly suggest that pesticide registration guidelines should expand to consider the toxicity of the formulated products, along with any surfactants or adjuvants that may be added if these chemicals are suggested on the herbicide label. Since active ingredients are not used alone, and neither are the additives, toxicity data should consider the possible synergistic effects of these chemicals and expand to require data for the tank-mix of formulated products. In short, the combination of chemicals which will be applied (formulated product with or without additives as required) should be what regulatory toxicity data represents.

CONCLUSION

Significant differences in phytotoxicity existed between the active ingredient glyphosate and one of its formulated products Round-Up Original[®] even though the effect of surfactant had been controlled for. These results indicate that the unknown “inert” ingredients contained in the formulated herbicide product enhanced its overall phytotoxicity. Based on these findings (and the limited data found in the literature), pesticide registration guidelines should require the tank-mix formulation that will actually be applied in the field when attempting to determine the phytotoxicity of any given herbicide.

Table 4-1. Inhibition concentrations (IC_p) calculated for each species and treatment expressed as g ai/ha. Confidence intervals are shown in brackets.

Species	Round-Up IC_{25}	Glyphosate IC_{25}	Round-Up IC_{50}	Glyphosate IC_{50}
<i>A. gerardii</i>	n/a	n/a	n/a	n/a
<i>G. max</i>	67 (37, 85)	110 (n/a)	111 (84, 132)	n/a
<i>L. canadensis</i>	115 (78, 142)	144 (80, 230)	189 (158, 289)	389 (294, 658)
<i>L. sativa</i>	112 (93, 138)	352 (282, 474)	223 (185, 313)	n/a
<i>S. lycopersicon</i>	51 (32, 68)	126 (n/a)	89 (60, 113)	n/a
<i>S. nigrum</i>	76 (65, 98)	49 (49, 52)	152 (126, 212)	100 (97, 104)
<i>V. americana</i>	111 (90, 171)	709 (356, 883)	227 (178, 343)	n/a
<i>Z. mays</i>	92 (74, 103)	n/a	105 (93, 120)	n/a

Table 4-2. Summary of the two-way ANOVAs comparing percent of control to treatment (glyphosate or Round-Up Original[®]) as well as dose for each of the species tested. For all species $n = 25$ (except for *S. nigrum*, $n = 15$).

Species	Factor	F - value	df	p - value
<i>A. gerardii</i>	Treatment	5.622	1	0.023
	Dose	4.613	1	0.004
	Interaction	0.740	4	0.570
<i>G. max</i>	Treatment	43.677	1	< 0.001
	Dose	12.57	1	< 0.001
	Interaction	2.74	4	0.042
<i>L. canadensis</i>	Treatment	16.854	1	< 0.001
	Dose	54.52	1	< 0.001
	Interaction	1.424	4	0.244
<i>L. sativa</i>	Treatment	170.73	1	< 0.001
	Dose	3.65	1	0.013
	Interaction	1.77	4	0.154
<i>S. lycopersicon</i>	Treatment	42.54	1	< 0.001
	Dose	22.52	1	< 0.001
	Interaction	4.17	4	0.006
<i>S. nigrum</i>	Treatment	17.78	1	< 0.001
	Dose	11.68	1	< 0.001
	Interaction	3.38	4	0.019
<i>V. americana</i>	Treatment	147.92	1	< 0.001
	Dose	7.96	1	< 0.001
	Interaction	2.77	4	0.04
<i>Z. mays</i>	Treatment	33.24	1	< 0.001
	Dose	21.80	1	< 0.001
	Interaction	14.57	4	< 0.001

Figure 4-1. Comparison of the effects of the formulated product Round-Up Original[®] and the active ingredient glyphosate on biomass (expressed as a percent of the controls) for eight different terrestrial plant species.

Figure 4-1.

GENERAL CONCLUSIONS

Four different experiments were conducted which investigated potential inadequacies highlighted in current pesticide registration guidelines. An overview of the potential weaknesses in current guidelines along with suggested improvements based on the results obtained from this thesis are shown in Table AA.

There are many uncertainties associated with plant species selection and herbicide regulatory guidelines. In general, no significant differences were found in herbicide sensitivity between the crop and non-crop species included in this study with the five herbicides tested. It was also shown that crops in which a specific herbicide is intended to be used, along with their wild relatives, have a higher tolerance to that herbicide than other species. Also noteworthy is that grasses were in general more tolerant to herbicide exposure than the broadleaf species tested. Based on these results, it seems that current pesticide registration guidelines are too rigid in terms of species selection, requiring corn and soybean, as well as three other monocotyledonous species to be included in every herbicide risk assessment regardless of known tolerances to certain herbicides. The strict species selection requirements of current pesticide registration guidelines, along with the data collected in this experiment brings up the following question: “should risk assessments include species that are known to not be at risk?” It is suggested that crop species in which the herbicide will be sprayed, as well as other species with a known tolerance (e.g. grasses) not be included in herbicide risk assessments that aim to predict the effects of any herbicide on non-target vegetation.

Significant differences in herbicide sensitivity between cultivars of the same crop species were found for all those included in this study. It is well known that differences

between varieties exist, but the results gathered in this experiment show that the variation among cultivars is great enough to alter the conclusions made in risk assessments using current regulatory protocol. It is suggested that if crop species continue to be utilized in regulatory phytotoxicity testing, that several cultivars of the same crop be included.

It was shown in the first experiment that no differences in herbicide sensitivity exist between the crops and non-crops tested, and in the second study that significant differences exist between cultivars of the same crop species. Based on these findings, can it be concluded that crop species are adequate representatives of the herbicide sensitivity found among all plant species?

To help answer the question posed above regarding crops as surrogates for all plant species in regulatory testing, the range in cultivar sensitivity for several crops was compared to the IC_{25} of the closest wild relative included in this experiment (Table BB). For atrazine, both lettuce and radish had ranges in sensitivity among cultivars that were below the IC_{25} of the wild species, indicating that despite variation within the crop varieties tested, the wild species would still have been protected regardless of the cultivar chosen. However, the IC_{25} of *S. nigrum* was found within the range of cultivar sensitivity and so depending on the cultivar chosen to include in risk assessment, this wild species may not necessarily have been protected. Similar results were found with MCPA, in that three of the four crops tested had an IC_{25} lower than their wild relatives. The only exception for this herbicide was *A. gerardii* whose IC_{25} was within the range of the corn cultivars and therefore depending on the corn variety chosen, this species may not have been protected. Interestingly, only *A. syriaca* would have been protected out of the four wild species potentially at risk to imazethapyr, since this was the only wild species whose

IC₂₅ was above the range of radish cultivar sensitivity. The wild relatives of lettuce, tomato, and corn were all sensitive at levels lower than the most sensitive cultivar of each crop.

Going back to the question “are crop species adequate representatives of the herbicide sensitivity of all plant species”, it seems that the answer is quite dependent on the herbicide in question, which cultivars are chosen to be included in the risk assessment, and the known tolerance level of the different species tested. Although it is important to protect agronomically important species from accidental herbicide drift, it is equally important to protect the habitats bordering areas where herbicides are used due to the ecological importance of the many wild plant species found there. Wild plant species from habitats most at risk to herbicide drift (such as those bordering agricultural fields) should be included in testing so that both types of plant species (crops and non-crops) are equally represented in risk assessment.

It was shown that chlorophyll fluorescence is an excellent tool for detecting early, non-visual herbicide effects in both crop and non-crop plant species. It was also found that chlorophyll fluorescence and visual rating can produce comparable predictions of phytotoxicity (based on IC₂₀ comparisons), although always an under-estimation, compared to biomass. Therefore, biomass should remain the preferred endpoint in pesticide registration guidelines with chlorophyll fluorescence and visual rating providing additional information that would be overlooked if biomass was relied upon exclusively.

Data collected during this thesis showed that significant differences in phytotoxicity exist between the herbicide active ingredient glyphosate and one of its formulated products Round-Up Original® when the addition of surfactant is taken into

account. Using current regulatory protocol, the phytotoxicity of the tank-mix product (Round-Up Original[®] plus recommended surfactant) would have been greatly underestimated and so it is suggested that pesticide registration guidelines expand to consider the toxicity of the complete formulated tank-mix that will be applied in the field.

The objective of this thesis was to determine whether current pesticide registration guidelines, specifically the components highlighted in each of the four experiments conducted, are adequate for the protection of non-target plant species and habitats. Several suggestions have been made to each area of the guidelines investigated that would improve current risk assessment protocol and aid in the better protection of non-target plant species. However, there is still much work to be done, especially in regards to species selection. Also important are experiments investigating differences that may exist between data produced in greenhouses compared to the field, single-species tests compared to multiple-species tests (micro- or mesocosms), and whether longer test periods would yield different results regarding phytotoxicity of any given herbicide.

Table AA. Areas of current pesticide registration guidelines which have been identified as potential weaknesses in regards to the protection of non-target plant species exposed to herbicides.

Highlighted Weakness in Current Guidelines	Current Protocol	Suggested Improvements
Species Selection	Crop species only	<ul style="list-style-type: none"> - Inclusion of crop and non-crop species - No species with known tolerance or in which the herbicide will be used be included
Cultivar Variability	One crop variety only	<ul style="list-style-type: none"> - Comparison of several crop cultivars
Endpoints	Usually biomass and perhaps a visual rating system	<ul style="list-style-type: none"> - Biomass should remain the preferred endpoint - Addition of chlorophyll fluorescence to detect early, non-visual effects and visual rating to provide information related to growth and development that biomass can not
Tested Chemical	Active ingredient only	<ul style="list-style-type: none"> - Toxicity tests using complete tank-mix formulation

Table BB. The range in sensitivity to each herbicide tested between five cultivars of different crop species compared to a closely related wild relative. Herbicide sensitivity shown as the 25% inhibition concentration (g ai/ha).

Herbicide	Label Rate (g ai/ha)	Crop Species	IC ₂₅ of the Most Sensitive Cultivar	IC ₂₅ of the Least Sensitive Cultivar	Closely Related Wild Relative	IC ₂₅ of the Wild Relative
Atrazine	1296	<i>Lactuca sativa</i>	30	66	<i>Lactuca canadensis</i>	97
		<i>Raphanus sativus</i>	81	122	<i>Asclepias syriaca</i>	130**
		<i>Solanum lycopersicon</i>	37	148	<i>Solanum nigrum</i>	67
Imazethapyr	91.2	<i>Lactuca sativa</i>	51	922*	<i>Lactuca canadensis</i>	7
		<i>Raphanus sativus</i>	4	17*	<i>Asclepias syriaca</i>	17
		<i>Solanum lycopersicon</i>	***	15	<i>Solanum nigrum</i>	3
		<i>Zea mays</i>	9	202*	<i>Zea mays</i>	4
MCPA	750	<i>Lactuca sativa</i>	4	26	<i>Lactuca canadensis</i>	44
		<i>Raphanus sativus</i>	7	32	<i>Asclepias syriaca</i>	126
		<i>Solanum lycopersicon</i>	17	44	<i>Solanum nigrum</i>	224
		<i>Zea mays</i>	325	2768	<i>Zea mays</i>	690

* herbicide doses tested did not elicit a 25% reduction in biomass compared to controls so 2x the highest doses tested was used as the IC₂₅

** herbicide doses tested resulted in 100% mortality despite the range-finding test so 50% of the lowest dose tested was used as the IC₂₅

*** herbicide doses tested resulted in 100% mortality despite range-finding test; 50% of the lowest dose tested results in a IC₂₅ higher than that estimated for the least sensitive cultivar. Therefore, the IC₂₅ can simply be estimated as < 15 g ai/ha.

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Appendix 1: Herbicides Selected for Phytotoxicity Testing

Five commercial herbicides containing different active ingredients with various modes of action were chosen for testing.

Herbicide Common Name	Herbicide Commercial Name	Content of Active Ingredient (g/L)	Selected Label Rate (g ai/ha)	Mode of Action
Atrazine	Aatrex Liquid 480	480	1296	PSII Inhibitor
Bentazon	Basagran	480	960	PSII Inhibitor
Glyphosate	Round-Up Original	356	2136	EPSP Inhibitor
Imazethapyr	Pursuit	240	91.2	ALS Inhibitor
MCPA	MCPA Amine 500	500	750	Synthetic Auxin

Atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine] is a selective systemic herbicide used for the pre- and post-emergence control of broadleaf weeds and grasses in agricultural and industrial applications (Thomson, 2002). In Ontario, atrazine was the second most commonly used agricultural herbicide in 2003 (McGee, 2004) applied mainly to corn but also used in low-bush blueberries and tolerant canola cultivars throughout Canada (OMAFRA, 2002; Syngenta, 2005). Atrazine is absorbed mainly through the roots, and secondarily through the foliage of the plant, then translocated via the xylem where it eventually accumulates in the leaves and apical meristems (Tomlin, 2003). Atrazine prevents electron transport and the transfer of light energy at the D-1 quinone-binding protein in photosystem II resulting in chlorosis and eventual plant death (Ashton and Crafts, 1973; Fuerst and Norman, 1991; Hess, 2000).

Bentazon [3-(1-methylethyl)-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide] is a post-emergence herbicide effective on broadleaf species only, most commonly used to

control weeds in soybeans, corn, dry beans, winter wheat, and certain fruits and vegetables in Ontario (BASF, 2002; McGee et al., 2004). In addition, bentazon is used throughout the world on many important crops, including cotton and rice (Cobb, 1992). A selective contact herbicide that is taken up via the foliage, bentazon targets photosystem II, acting as a relatively weak inhibitor of electron flow at the D1 polypeptide (Cobb, 1992; Tomlin, 2003). Bentazon is a benzothiadiazole compound whose efficacy is improved with increasing temperatures (Thomson, 2002).

Glyphosate [N-(phosphonomethyl) glycine] is a non-selective systemic herbicide used for the control of annual and perennial grasses and broad-leaved plant species, as well as woody brush and trees, in agricultural, industrial, and domestic situations (Monsanto Canada Inc., 2001; Tomlin, 2003). In Ontario, total glyphosate usage for agricultural purposes has increased 58% since 1998 (McGee et al., 2004), which could most likely be attributed to the increasing use of genetically modified crops exhibiting glyphosate resistance. Glyphosate inhibits 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) which results in an accumulation of shikimate-3-phosphate, eventually preventing aromatic amino acid production, and thus effecting protein synthesis; other physiological and chemical effects have also been noted (Baylis, 2000).

Imazethapyr [(+/-)-2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-5-ethyl-3-pyridinecarboxylic acid] is used mainly on soybeans in Ontario, but can also be used on other leguminous crops (OMAFRA, 2002) for the control of a total of 14 different broadleaf and grass weed species from eight families on the Canadian label (Cyanamid, 2004, now BASF Inc.). This herbicide is absorbed by the roots and foliage of the target plant species and is systemically translocated throughout

the tissues where, due to inhibition of the enzyme acetolactase synthase (ALS), the production of the amino acids valine, leucine, and isoleucine is prevented eventually causing disruption of protein and DNA synthesis (Shaner et al., 1984; Tomlin, 2003).

MCPA [(4-chloro-2-methylphenoxy)acetic acid] is a selective systemic herbicide that accumulates in the meristematic regions of target plants, where it disrupts hormone balances, protein synthesis, and plant growth by acting as a synthetic auxin (Tomlin, 2003). The specific site of action of this herbicide is not known; there are potentially multiple sites of action resulting in the herbicidal activity of this chemical (Gunsolus and Curran, 2002). This herbicide is used for the post-emergence control of approximately 74 broadleaf weed species from 19 different families in Canadian cereal crops (United Agri Products, 1999).

Appendix 2: Germination Requirements for Non-crop Species

Species	English Common Name	Family	Species Type ^a	Lifespan ^b	Time to 50% Germination (days)	Temp. (°C)	Photoperiod ^c
<i>Asclepias syriaca</i>	Common Milkweed	Asclepiadaceae	D	A	4 - 6	n/a	L > D
<i>Fragaria virginiana</i>	Thick-leaved Wild Strawberry	Rosaceae	D	P	10 - 14	25	L > D
<i>Helianthus strumosus</i>	Rough-leaved Sunflower	Asteraceae	D	P	7 - 10	21	n/a
<i>Lactuca canadensis</i>	Tall Lettuce	Asteraceae	D	B, A	7 - 10	21	L > D
<i>Solanum nigrum</i>	Black Nightshade	Solanaceae	D	A	10 - 14	21	L > D
<i>Vicia americana</i>	American Vetch	Fabaceae	D	P	7	21	n/a
<i>Andropogon gerardii</i>	Big Bluestem	Poaceae	M	P	3 - 5	25	L > D
<i>Elymus canadensis</i>	Canada Wild Rye	Poaceae	M	P	3 - 4	alternating 24/15	n/a
<i>Poa compressa</i>	Canada Bluegrass	Poaceae	M	P	7	21	L > D

Species	Special Treatments ^d	Sensitivities ^e	Germination Rate (%)
<i>Asclepias syriaca</i>	V	TA	70 - 80
<i>Fragaria virginiana</i>	nil	TA	75 - 80
<i>Helianthus strumosus</i>	B, S	TA	10 - 20
<i>Lactuca canadensis</i>	LS	TA	75 - 80
<i>Solanum nigrum</i>	nil	TA	75 - 80
<i>Vicia americana</i>	SC, ST	TA	50 - 60
<i>Andropogon gerardii</i>	GA	PM	95 - 100
<i>Elymus canadensis</i>	B	PM	95 - 100
<i>Poa compressa</i>	GA	PM	95 - 100

*Note: footnotes corresponding to *a - e* are found on the following page.

Footnotes corresponding to Appendix 2:

- a) **D** = dicotyledon; **M** = monocotyledon
- b) **A** = annual; **B** = biennial; **P** = perennial
- c) **L > D** = more hours of sunlight (natural or artificial) than darkness are required for germination to occur
- d) **B** sow seeds approximately 10 mm under soil surface
 - GA** mix seeds with equal part sand in a covered Petri dish and moisten with 500 ppm Gibberellic acid for 24 hours at room temperature; surface sow on moist 2:1 ProMix to sand mixture
 - LS** mix seeds with equal part moist vermiculite and stratify for 90 days in darkness at 4°C; surface sow seeds on moist 2:1 ProMix to sand mixture
 - S** mix seeds with equal part moist vermiculite and stratify for 4 – 6 weeks in darkness at 4°C; surface sow seeds on moist 2:1 ProMix to sand mixture
 - SC** scarify seeds using 100 grade sandpaper to remove seedcoat; fill shallow tray ~ 75% with moist ProMix and cover surface with a layer of fine sand; place seeds on sand and cover lightly with moist ProMix
 - ST** staking required
 - V** mix seeds with equal part moist vermiculite and stratify for 30 days in darkness at 4°C; surface sow seeds on moist vermiculite
- e) **PM** susceptible to Powdery Mildew – do not overwater
 - TA** susceptible to both aphids and thrips; control using sticky traps, predatory mites, and ladybug beetles

Appendix 3: Herbicide Definitive Doses Applied to Each Species

Herbicide Common Name	Herbicide Commercial Name	Label Rate (g ai/ha)	Species	Range of Doses Tested (g ai/ha)
Atrazine	Aatrex Liquid 480	1296	<i>A. gerardii</i>	648, 972, 1465, 2190, 3279
			<i>A. syriaca</i>	50, 58, 66, 76, 87
			<i>A. sativa</i>	1166, 1750, 2631, 3940, 5910
			<i>E. canadensis</i>	65, 104, 168, 259, 428
			<i>F. ananassa</i>	26, 39, 65, 91, 130
			<i>F. virginiana</i>	26, 39, 65, 91, 130
			<i>G. max</i>	259, 350, 467, 635, 855
			<i>H. annuus</i>	194, 259, 324, 428, 557
			<i>H. strumosus</i>	13, 26, 39, 78, 130
			<i>L. canadensis</i>	13, 26, 39, 78, 130
			<i>L. sativa</i>	104, 130, 168, 207, 259
			<i>P. compressa</i>	65, 117, 181, 324, 544
			<i>R. sativus</i>	259, 324, 402, 505, 635
			<i>S. lycopersicon</i>	130, 168, 220, 285, 376
			<i>S. nigrum</i>	130, 194, 272, 389, 570
			<i>T. aestivum</i>	324, 376, 428, 492, 570
			<i>V. americana</i>	389, 505, 661, 855, 1115
Bentazon	Basagran	960	<i>A. gerardii</i>	960, 1440, 2160, 3245, 4858
			<i>A. syriaca</i>	480, 605, 749, 941, 1171
			<i>A. sativa</i>	960, 1440, 2160, 3245, 4858
			<i>E. canadensis</i>	960, 1440, 2160, 3245, 4858
			<i>F. ananassa</i>	480, 720, 1085, 1622, 2429
			<i>F. virginiana</i>	134, 173, 211, 259, 326
			<i>G. max</i>	960, 1440, 2160, 3245, 4858
			<i>L. canadensis</i>	48, 96, 173, 326, 624
			<i>L. sativa</i>	960, 1056, 1162, 1277, 1402
			<i>P. compressa</i>	960, 1440, 2160, 3245, 4858
			<i>R. sativus</i>	240, 298, 374, 470, 586
			<i>S. lycopersicon</i>	960, 1440, 2160, 3245, 4858
			<i>S. nigrum</i>	960, 1440, 2160, 3245, 4858
			<i>T. aestivum</i>	960, 1440, 2160, 3245, 4858

Herbicide Common Name	Herbicide Commercial Name	Label Rate (g ai/ha)	Species	Range of Doses Tested (g ai/ha)
Bentazon	Basagran	960	<i>A. gerardii</i>	648, 972, 1465, 2190, 3279
			<i>V. americana</i>	960, 1440, 2160, 3245, 4858
			<i>Z. mays</i>	960, 1440, 2160, 3245, 4858
Glyphosate	Round-Up Original	2136	<i>A. gerardii</i>	21, 32, 49, 73, 109
			<i>A. syriaca</i>	64, 85, 107, 150, 192
			<i>A. sativa</i>	171, 256, 384, 577, 876
			<i>E. canadensis</i>	21, 43, 64, 85, 107
			<i>F. ananassa</i>	43, 64, 85, 107, 171
			<i>F. virginiana</i>	214, 320, 491, 726, 1089
			<i>G. max</i>	43, 64, 85, 107, 171
			<i>L. canadensis</i>	43, 85, 192, 427, 983
			<i>L. sativa</i>	320, 384, 470, 555, 662
			<i>P. compressa</i>	22, 43, 64, 107, 171
			<i>R. sativus</i>	22, 43, 65, 85, 150
			<i>S. lycopersicon</i>	22, 43, 64, 107, 171
			<i>S. nigrum</i>	171, 256, 384, 577, 876
			<i>T. aestivum</i>	43, 64, 85, 107, 171
			<i>V. americana</i>	214, 320, 449, 641, 940
			<i>Z. mays</i>	64, 85, 107, 150, 192
Imazethapyr	Pursuit	91.2	<i>A. gerardii</i>	1.9, 3.6, 5.5, 10.9, 19.2
			<i>A. syriaca</i>	54.7, 65.7, 78.4, 94.9, 113.1
			<i>A. sativa</i>	9.1, 16.4, 29.2, 52.9, 94.7
			<i>E. canadensis</i>	45.6, 59.3, 77.5, 100.3, 130.4
			<i>F. ananassa</i>	1.8, 2.7, 4.6, 6.4, 9.1
			<i>F. virginiana</i>	9.1, 16.4, 29.2, 52.9, 95.8
			<i>G. max</i>	91.2, 136.8, 205.2, 308.3, 461.5
			<i>L. canadensis</i>	0.91, 1.8, 2.7, 5.5, 9.1
			<i>L. sativa</i>	91.2, 137, 205, 308, 461
			<i>P. compressa</i>	60.2, 65.7, 72.1, 79.3, 86.6
			<i>R. sativus</i>	1.8, 2.7, 3.6, 5.5, 8.2
			<i>S. lycopersicon</i>	45.6, 54.7, 65.7, 78.4, 94.8
			<i>S. nigrum</i>	0.91, 1.8, 2.7, 4.6, 7.3
			<i>T. aestivum</i>	7.3, 10.9, 15.5, 21.9, 31.9
			<i>V. americana</i>	91.2, 137, 205.2, 308.3, 461.5
			<i>Z. mays</i>	23, 33, 48, 69, 101

Herbicide Common Name	Herbicide Commercial Name	Label Rate (g ai/ha)	Species	Range of Doses Tested (g ai/ha)
MCPA	MCPA Amine 500	750	<i>A. gerardii</i>	750, 1125, 1688, 2535, 3795
			<i>A. syriaca</i>	375, 473, 585, 735, 915
			<i>A. sativa</i>	750, 1125, 1688, 2535, 3795
			<i>E. canadensis</i>	750, 1125, 1688, 2535, 3795
			<i>F. ananassa</i>	75, 120, 195, 308, 495
			<i>F. virginiana</i>	300, 375, 473, 585, 735
			<i>G. max</i>	75, 113, 173, 255, 383
			<i>L. canadensis</i>	75, 113, 173, 255, 383
			<i>L. sativa</i>	8, 15, 23, 38, 60
			<i>P. compressa</i>	750, 1125, 1688, 2535, 3795
			<i>R. sativus</i>	15, 23, 30, 45, 68
			<i>S. lycopersicon</i>	38, 68, 128, 240, 443
			<i>S. nigrum</i>	450, 518, 593, 683, 788
			<i>T. aestivum</i>	750, 1125, 1688, 2535, 3795
			<i>V. americana</i>	75, 105, 135, 188, 248
			<i>Z. mays</i>	750, 1125, 1688, 2535, 3795