Chapter 14

Assessment of Wild Mustard (*Sinapis arvensis* L.) Resistance to ALS-inhibiting Herbicides

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Additional information is available at the end of the chapter

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Abstract

There is an urgent need for rapid, accurate, and economical screening tests that can determine if weeds surviving a herbicide application are resistant. This chapter describes development and application of a simple root length bioassay technique for detection of wild mustard (*Sinapis arvensis* L.) resistance to ALS-inhibiting herbicides. This bioassay was performed in 2-oz WhirlPak® bags filled with 50 g of soil wetted to 100% moisture content at field capacity. Wild mustard seeds were pre-germinated in darkness in Petri dishes lined with moist filter paper for 2 days. Six seeds with well-developed radicles were planted in the non-treated soil and in soil with added herbicide, and plants were grown in a laboratory under fluorescent lights. After 4 days of growth, WhirlPak® bags were cut open, soil was washed away, intact plants were removed, and root length was measured with a ruler. The concentration of each herbicide in soil at which a significant root inhibition of susceptible biotype, but no root inhibition of a resistant biotype occurred was selected. Susceptibility/resistance of wild mustard populations was estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil as compared to the plants grown in the non-treated soil.

Keywords: Herbicide resistance, wild mustard, ALS-inhibiting herbicides, bioassay

1. Introduction

Repeated applications of herbicides with the same mode of action have resulted in weeds developing resistance. Herbicide resistance in weeds refers to the inherited ability of a weed biotype to survive a herbicide application to which the original population was susceptible. It is not a genetic change caused by herbicides that allows resistance to develop. The resistant biotype is present in low numbers in natural populations, and when a herbicide is applied, most of the susceptible weeds die but the few resistant weeds survive, mature, and produce

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seed. If the same herbicide continues to be applied and the resistant weeds reproduce, the percentage of the weed population that is resistant increases [1]. The risk of weeds developing resistance is particularly high in production of herbicide-resistant crops where only one or two herbicide modes of action can be applied, or are applied due to economic and convenience factors, for weed control. Development of resistance may lead to economic losses because of the lack of alternative herbicide choices [2].

Acetolactate synthase (ALS)-inhibiting herbicides have been used extensively in agricultural production mainly because of their remarkable efficacy at very low application rates. However, it has been recognized that the ALS-inhibitors are the most resistance-selective herbicide group. ALS-herbicides were first introduced in the early 1980s, and since then, rapid increase in incidence of resistance to these herbicides has been reported; more weeds have become resistant to ALS-inhibiting herbicides than to any other herbicide mode of action [1-3].

There is an urgent need for tests that can determine if weeds surviving a herbicide application are resistant. However, before assuming that weeds are resistant because they were not controlled, other factors that might affect herbicide performance, such as misapplication, unfavorable weather conditions, improper timing of herbicide application, and weed flushes after application of a nonresidual herbicide need to be considered. If resistance is occurring, the problem needs to be identified as early as possible as losses of herbicide options could have important economic and environmental consequences to agricultural production especially if herbicide cross-resistance or multiple resistance occur [1].

Various techniques have been proposed for confirming ALS-inhibitor resistance in weed populations. The whole-plant pot (soil) assay conducted in a greenhouse (ca. 4–6 week duration) is the most frequently used method for identifying herbicide-resistant weeds, as results are considered most relevant to field conditions [4]. Nevertheless, a number of rapid, soil-less (dish) bioassays have been developed over the past 25 years to reliably discriminate herbicide-resistant from herbicide-susceptible weeds, such as various 7-day acetyl CoA carboxylase inhibitor-resistant bioassays [4, 5]. However, dish assays have not been successful to date in reliably discriminating between ALS inhibitor-resistant and susceptible weeds. Validated rapid tests for resistance to herbicides with this mode of action would be less expensive than pot assays and allow for a quicker turnaround time to clients, thereby facilitating proactive and timely implementation of resistance management by producers. Additionally, molecular techniques are increasingly being used in testing laboratories to confirm ALS-inhibitor resistance, as target-site (*ALS*) mutation is the most common mechanism of herbicide resistance in broadleaf weeds [6]. However, cost of equipment and testing (multiple mutations can confer *ALS* resistance) may be prohibitive, and negative results cannot exclude the existence of a different possible mechanism of resistance.

Wild mustard (*Sinapis arvensis* L.) is a common weed in field crops in the Canadian prairies. It ranked 11th of 101 weeds in a 2002 Manitoba survey of cereal and oilseed crops; and 15th of 124 weeds in a 2003 Saskatchewan survey of cereal, oilseed, and pulse crops [7]. Herbicide resistance to ALS-inhibiting herbicides has been reported for a number of populations of wild mustard in Canada [8]. Herbicide-resistant populations were first reported in Manitoba in 1992 [9], Alberta in 1993 [10], and Saskatchewan in 2002 [11]. Based

on samples submitted by growers between 2007 and 2011, 16 wild mustard populations from Saskatchewan were confirmed as ALS inhibitor-resistant, compared with 12 populations between 2002 and 2006 [5].

This chapter describes development and application of a rapid and simple root length bioassay technique for assessment of wild mustard susceptibility/resistance to selected ALS-inhibiting herbicides.

2. Development of a wild mustard bioassay

ALS-herbicides inhibit biosynthesis of branched-chain amino acids and affect primarily root growth of susceptible plants through inhibition of cell division at the root tips. Therefore, measuring root length reduction of sensitive plant species is the most common detection approach used in bioassays for ALS-inhibiting herbicides [12-18].

2.1. Wild mustard biotypes

Seeds of 15 wild mustard biotypes that were collected in western Canada and characterized as ALS herbicide-susceptible and herbicide-resistant based on pot assays were obtained from Agriculture and Agri-Food Canada (AAFC) in Saskatoon, SK (Table 1).

Seed germination was tested in Petri dishes lined with moist filter paper in darkness. After 1 day, only a few seeds germinated; after 2 days, the germination rates varied among biotypes from approximately 2 to 55% (Table 1). Two biotypes that had the highest germination rate, i.e., BT1 and BT7, were selected for further testing for the bioassay development.

2.2. Soil used for the bioassay

Soil used for the bioassay was collected from the lower slope position in a farm field (legal location SW36-20-4-3) near Central Butte, SK, Canada. The soil had the following selected properties: 2.2% organic carbon content, soil pH 6.9, 38% clay content, and 18% moisture content at field capacity.

2.3. Bioassay technique

Bioassay was performed in 57-g (2-oz) WhirlPak® bags [15]. A quantity of 50 g of soil was wetted to 100% moisture content at field capacity by adding 9 mL of water to soil; then soil was hand-mixed in a plastic dish and transferred to a WhirlPak® bag. Soil in the bag was gently packed to form a layer approximately 8 cm high, 6 cm long, and 1 cm wide. Six seeds were planted at a 2-mm depth and the soil surface was covered with a 5-mm layer of plastic beads to reduce soil drying (Figure 1). Plants were grown in the laboratory under fluorescent lights that had photosynthetic photon flux density of approximately 16 $\mu mol/m^2/s$ at the plant level, and plants were watered daily to 100% field capacity by adding water to a predetermined weight. At harvest time, intact plants were recovered

Wild mustard biotype	Susceptibility/resistance to selected ALS-inhibiting herbicides	Approximate germination rate (%)
BT1	Susceptible to tribenuron/thifensulfuron	55
BT2	Susceptible to tribenuron/thifensulfuron	24
BT3	Susceptible to tribenuron/thifensulfuron	3
BT4	Susceptible to imazethapyr	4
BT5	Susceptible to imazethapyr Susceptible to imazethapyr/imazamox	15
BT6	Susceptible to tribenuron/thifensulfuron	4
BT7	Strong resistance to ethametsulfuron Strong resistance to tribenuron/thifensulfuron	50
BT8	89% resistant to imazethapyr	8
BT9	100% resistant to imazethapyr/imazamox 100% resistant to tribenuron/thifensulfuron	23
BT10	100% resistant to imazethapyr/imazamox 100% resistant to tribenuron/thifensulfuron	15
BT11	90% resistant to imazethapyr/imazamox 50% resistant to tribenuron/thifensulfuron	9
BT12	100% resistant to imazethapyr/imazamox 100% resistant to imazethapyr 100% susceptible to tribenuron/thifensulfuron	16
BT13	100% resistant to imazamox 100% resistant to imazamox	7
BT14	100% resistant to imazethapyr/imazamox	2
BT15	98% resistant to imazethapyr 8% resistant to tribenuron/thifensulfuron	15

Table 1. Susceptibility/resistance of wild mustard biotypes to selected ALS-inhibiting herbicides evaluated in pot assays, and approximate seed germination rates.

from soil after the WhirlPak® bag was opened, and soil was washed away with water. After removal of plants, root length was measured with a ruler (Figure 2). This bioassay technique has been shown to be very useful for detecting ALS-inhibiting herbicides in soil with oriental mustard (*Brassica juncea* L.), primarily because plants with intact roots can be easily retrieved from soil and then measured [15, 16].

Figure 1. Mustard bioassay performed in WhirlPak® bags.

Figure 2. Intact mustard plants after removal from untreated soil.

2.4. Establishing conditions for growing wild mustard

The ideal root length of plants grown in a 57-g (2-oz) WhirlPak® bag is around 8 cm because of the 8-cm approximate height of soil in the bag. Root development beyond this height is obstructed as roots grow along the bottom of the bag and typically smaller increases in root elongation are observed at this point. In a root length bioassay, it is important that the measured root length reduction is in response to the herbicide of interest and not to other factors.

To establish the optimal duration of growth for wild mustard, two biotypes, i.e., BT1 and BT7, were grown from 2 to 6 days after seeding. Because of the low germination rates for most of the wild mustard populations, seeds were pregerminated for 2 days, and six seeds with well-developed radicles were transferred to soil. Root length increased with the duration of plant growth and was the highest after approximately 4–5 days (Figure 3). Growing plants longer did not increase root length, and a 4-day plant growth was selected for the wild mustard root length bioassay.

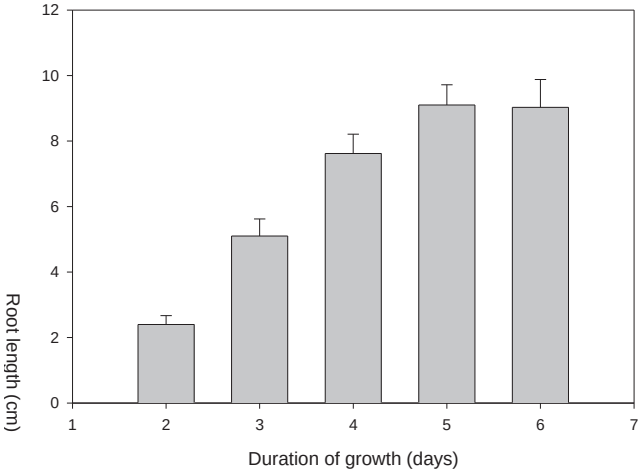

Figure 3. Root length of wild mustard grown from 2 to 6 days after seeding (each data point represents mean ± standard deviation).

3. Wild mustard response to selected ALS-inhibiting herbicides

Wild mustard response to four ALS-inhibiting herbicides was assessed. The selected ALS-herbicides were: flucarbazone – a sulfonylaminocarbonyltriazolinone (SCT) herbicide, pyroxsulam – a triazolopyrimidine (TP) herbicide, imazamox/imazethapyr – an imidazolinone (IMI) herbicide, and metsulfuron – a sulfonylurea (SU) herbicide.

3.1. Solution preparation and soil spiking

Technical-grade flucarbazone (99.1% pure) (from Bayer Co.), commercial formulation Simplicity containing pyroxsulam at a concentration of 30 g ai/L (from Dow AgroSciences Co.), commercial formulation Odyssey containing 35% imazamox and 35% imazethapyr (from BASF Co.), and technical-grade metsulfuron (93% pure) (from du Pont Inc.) were used for preparing stock solutions of each herbicide. The weighed quantity of a herbicide was transferred to a 1-L flask with 100 mL of methanol or acetone, and the flask was filled with water. A series of standard solutions in a concentration range from 0 to 1.5 ppm flucarbazone, 0 to 0.345 ppm pyroxsulam, 0 to 2.24 ppm imazamox/imazethapyr, and 0 to 0.32 ppm metsulfuron were prepared.

Soil was spiked with an ALS-inhibiting herbicide by first combining a 0.5-mL volume of a herbicide standard solution with 8.5-mL volume of water (for a total volume of 9 mL) and then transferring this solution to 50 g of soil yielding herbicide concentration from 0 to 15 ppb

flucarbazone, 0 to 3.45 ppb pyroxsulam, 0 to 22.4 ppb imazamox/imazethapyr, and 0 to 3.2 ppb metsulfuron. These concentration ranges were equivalent to field application rates from 0 to approximately 1X. Soil was then mixed, transferred to a WhirlPak® bag, and bioassay was performed as described above. After removing plants from soil, root length was measured, and root length inhibition (RLI %) was calculated using the formula [17]:

$$RLI(\%) = \left(1 - \frac{L_t}{L_0}\right) \times 100\%$$

where L_t is the root length in the herbicide-treated soil and L_0 is the root length in the untreated (control) soil.

3.2. Dose–response curves

To select a concentration of each herbicide in soil at which a significant root reduction of susceptible biotype, but no root reduction of a resistant biotype is observed, dose–response curves were constructed for representative susceptible and resistant seed samples. Based on the available susceptibility/resistance data (Table 1), biotypes were selected for assessment of wild mustard response to the four ALS-inhibiting herbicides. For the wild mustard biotypes that showed root length inhibition to a herbicide in a selected concentration range, dose–response curves were constructed by graphing root length inhibition data versus herbicide concentration in soil using a log-logistic model [19]:

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^b}$$

where C is the lower limit of the curve, D is the upper limit of the curve, b is the slope of the curve around GR_{50} value, and GR_{50} is the concentration corresponding to 50% inhibition. For the wild mustard samples that showed zero or near-zero root length inhibition to a herbicide in a selected concentration range, linear regression was used. A WhirlPak® bag seeded with plants was a replicate and each measurement was replicated four times.

As can be seen from Fig. 4, the selected susceptible biotypes showed root length inhibition to flucarbazone, pyroxsulam, imazamox/imazethapyr, and metsulfuron. Resistant biotypes did not exhibit sensitivity to these herbicides and root length inhibition was zero or near-zero in the concentration ranges tested. Root length inhibition of susceptible biotypes was approximately 40% in response to 15 ppb flucarbazone, 70% in response to 3.45 ppb pyroxsulam, 60% in response to 22.4 ppb imazamox/imazethapyr, and 70% in response to 3.2 ppb metsulfuron. Thus, testing susceptibility/resistance of wild mustard populations to the ALS-inhibiting herbicides can be accomplished by growing mustard plants in the herbicide-treated soil at the above concentrations. If the root length reduction is observed at these herbicide concentrations

as compared to the root length in the untreated soil, the wild mustard biotype is susceptible, while no root length reduction indicates resistance.

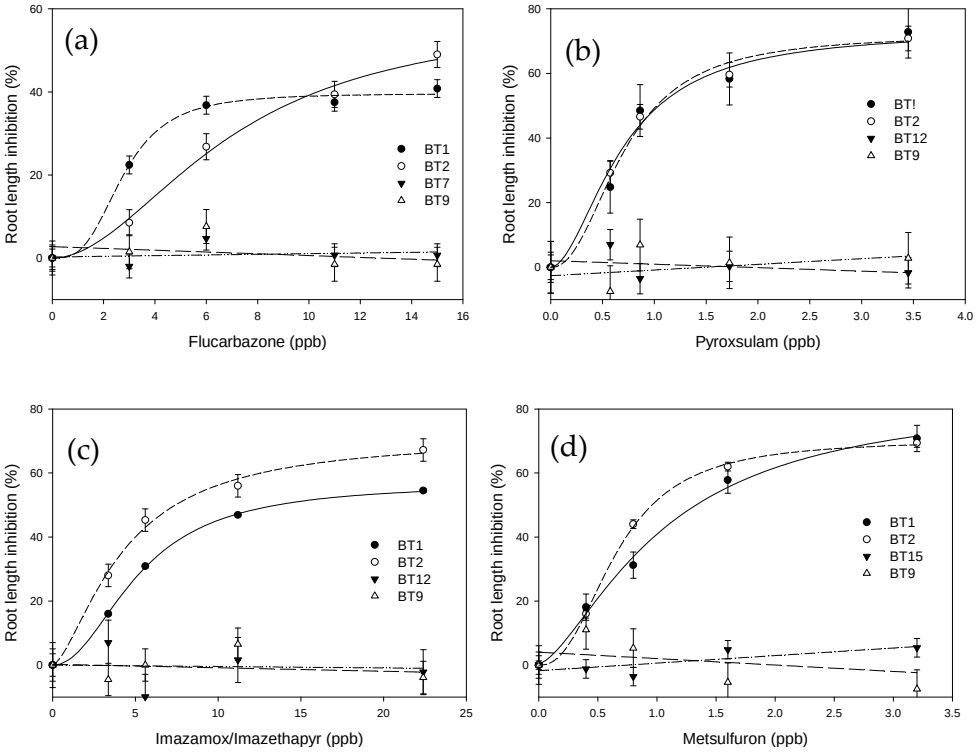

Figure 4. Dose–response of wild mustard to (a) flucarbazone, (b) pyroxsulam, (c) imazamox/imazethapyr, and (d) metsulfuron determined by the root length inhibition bioassay.

Percent resistance in the wild mustard populations was estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil (Table 2). This approach is particularly useful for biotypes that have variable root length in the untreated soil so that the percentage of uninhibited roots in the treated soil can be corrected. Typically, in the nontreated soil most of the wild mustard biotypes had roots that were approximately 7.5 ± 2.5 cm long and some short roots that had length less than 5 cm. Therefore, it is important that a wild mustard biotype being tested for susceptibility/resistance by this bioassay technique is grown both in the nontreated soil and in the herbicide-treated soil so that short roots obtained in the herbicide-treated soil would not be interpreted as herbicide-susceptible. Estimated percentages of resistant plants (Table 2) are in a very good agreement with the susceptibility/resistance data from the pot assays conducted in the greenhouse by AAFC Saskatoon (Table 1). Thus, these results show that the simple and rapid (6-day) root length bioassay performed

in a laboratory can be used in place of the whole-plant pot assay that requires ca. 4-6 weeks for the assessment of wild mustard resistance to ALS-inhibiting herbicides [4].

Wild mustard biotype	% resistant plants[a]			
	Flucarbazone	Pyroxsulam	Imazamox/ imazethapyr	Metsulfuron
BT1	0	0	0	0
BT2	0	0	0	0
BT3	60	0	0	0
BT4	100	10	15	15
BT5	0	0	0	0
BT6	0	0	0	0
BT7	100	-		-
BT8	100	100	100	0
BT9	100	100	100	100
BT10	100	100	100	100
BT11	100	100	100	100
BT12	100	100	100	0
BT13	100	100	100	0
BT14	100	100	100	100
BT15	100	100	100	70

[a] % resistant plants = number of uninhibited roots/total number of roots × 100 % in response to flucarbazone at 15 ppb, pyroxsulam at 3.45 ppb, imazamox/imazetapyr at 22.4 ppb, and metsulfuron at 3.2 ppb.

Table 2. Estimated percentage of resistant plants in wild mustard populations in response to ALS-inhibiting herbicides.

4. Conclusions

- The root length bioassay is suitable for assessment of susceptibility/resistance of wild mustard populations to ALS-inhibiting herbicides.
- To perform this bioassay, no specialized equipment is required and the bioassay is completed in 6 days. Seeds are pregerminated for 2 days, and plants are grown for 4 days in a laboratory under fluorescent light in plastic bags filled with untreated soil and herbicide-treated soil (15 ppb flucarbazone, 3.45 ppb pyroxsulam, 22.4 ppb imazamox/imazethapyr, or 3.2 ppb metsulfuron). Removal of plants from soil with water allows for recovery of intact roots that can be easily measured.

- Due to variability in root growth, a minimum of four replications of plants grown in the untreated and in herbicide-treated soil, i.e., a total of eight WhirlPak® bags seeded with plants are recommended.
- Based on root length in the untreated soil and in the herbicide-treated soil, susceptible and resistant wild mustard populations can be identified. Typically, susceptible wild mustard biotypes have RLI (%) of approximately 40% to 15 ppb flucarbazone, 70% to 3.45 ppb pyroxsulam, 60% to 22.4 ppb imazamox/imezathapyr, and 70% to 3.2 ppb metsulfuron, while RLI (%) for resistant wild mustard is near-zero.
- Alternatively, susceptibility/resistance may be estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil as compared to the plants grown in the untreated soil.
- Testing susceptibility/resistance to herbicides from each class of the ALS-inhibitors is required as wild mustard biotypes may be resistant to one class but susceptible to another.

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