Abstract

Application of a series of sublethal rates of diclofop-methyl (DM) herbicide to cloned individuals of Lolium multiflorum Lam increased their level of resistance to this herbicide. Under controlled environmental and field conditions, individuals of L. multiflorum genotypes herbicide-acclimated with 35 and 70 g a.i. ha\(^{-1}\) diclofop-methyl rates showed up to 80% survival and produced more aboveground biomass when exposed to four times higher rates compared to herbicide non-acclimated plants. The acquired increase in individual herbicide tolerance was not transmitted to their offspring, denoting the nature of acclimation response.

The lack of transmission of resistant traits to the progeny highlights that exposure to sublethal rates of herbicide in susceptible individuals allowed for the expression of pre-existing cytoplasmatic information or simultaneously induced and selected extra-nuclear contents associated with resistance. The potential mechanisms through which induced tolerance may occur in L. multiflorum plants treated with sublethal concentrations of diclofop-methyl are discussed.

Any recommendation or attempt to control weeds by reducing herbicide rates to increase economic returns and reduce the risk of environmental contamination should (a) acknowledge the potential acclimation responses in target plants and (b) be made and based on long-term studies.

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Keywords: Diclofop-methyl; Herbicide acclimation; L. multiflorum; Sublethal dosages

1. Introduction

Weed control in modern agriculture includes a range of physical and cultural practices such as cultivation, burning, crop rotations and grazing. However, due to agronomic and economic reasons, herbicides have been adopted as the main weed control tool. The extensive usage of herbicides is better perceived when compared to other pesticides: whereas the amount of herbicides used during 1997 in USA represented almost 65% of the total pesticides used in major crops (National Research Council, 2000), herbicides represented 70 and 50% of pesticide sale markets in Argentina and Australia, respectively (Alvarez, 1998; Radcliffe, 2002).
The use of herbicides to control 100% of weeds infesting cropping fields is neither economical nor practically achievable: a paradox is found between the high efficiency of herbicides as “killing compounds” and the largely low efficiency of its application (Wauchope et al., 1997; Kudsk and Streibig, 2003). For example, recommendations about the use of herbicide rates are based on weed growth stages found at spray time. However, it is highly unlikely to find uniform plant sizes or phenological stages within weed populations. This source of variability leads to a variation in the quantity of active ingredient that arrives by unit weight or leaf area of target plants. Large plants produce a “diluting effect” of the herbicide, promoting sublethal conditions and leading to poor weed controls (Wauchope et al., 1997; Vila-Aiub et al., 2003a).

Present cropping systems rely on the selection pressure or efficacy exerted by herbicides to reduce successfully weed infestations (Gressel and Segel, 1990; Jasieniuk et al., 1996). A combination of various elements determines the strength of herbicide selection: (a) the dosage of the herbicide, (b) the growth stage of plants and (c) the environmental conditions (air temperature, light and plant water status among others) that determine the dimension of contact, entry and uptake of herbicides by plants (Zimdahl, 1993). Holding all factors comparable, a variation in the rate or dosage of the herbicide dramatically changes the intensity of selection pressure over the target populations. The explanation for this phenomenon is based on the fact that a quantitative variation is inherent in the susceptibility of plant species. For example, a susceptible *Lolium rigidum* population exposed to glyphosate at 450 g.a.i. ha⁻¹ showed none or few surviving plants. However, the same population exposed to lower rates showed 20% survival (Powles et al., 1998; Lorraine-Colwill et al., 2002). The same pattern of increasing survival when herbicide rates are reduced has been observed in several weed species selected with different classes of herbicides (Bravin et al., 2001; Hidayat and Preston, 2001; Walsh et al., 2001).

There is empirical evidence supporting the idea that “susceptible” weed populations, despite the lack of mechanisms that enable them to evolve towards resistance when exposed to high herbicide rates (Heag and LeBaron, 2001) possess the ability to tolerate herbicide doses below the manufacturer recommended commercial rates (i.e. sublethal doses). There are a number of non-mutually exclusive explanations to consider when plants are submitted to sublethal herbicide rates: (a) the inhibition of plant metabolism is not high enough to lead to plant mortality (Tardif et al., 1996; Hidayat and Preston, 2001), (b) enzyme-mediated detoxification and sequestration of small amounts of active compounds is likely to happen, (c) association with symbiotic *Neotyphodium* fungal endophytes that may promote a weak tolerance mechanism (Vila-Aiub and Ghersa, 2001; Vila-Aiub et al., 2003a) and (d) chance.

This issue becomes radically important since a number of events reduce herbicide dosages from those rates recommended during the spray at farming conditions. Reduced herbicide doses can come about by either (a) farmers cutting rates, (b) herbicide drift by windy conditions or (c) heterogeneity associated with the size of target plants that makes bigger plants receive less effective dose compared to smaller plants or (d) intrinsic variability associated with herbicide applications on weed canopies (Caseley and Walker, 1990; Gressel, 1995; Wauchope et al., 1997).

Intentional reduction of herbicide dosages for economical and manageable reasons, and/or environmental concerns, is being carried out by farmers in Europe and India (Gressel, 1995; Kudsk and Streibig, 2003). In recent years, efforts to increase economic returns and environmental benefits have pushed researchers to concentrate on the risks and efficacy of reducing herbicide rates to control weed populations (see review by Zhang et al., 2000). In some cases, single or sequential (split) applications with reduced rates can provide equal crop yields and higher net incomes than single applications of the full recommended herbicide rate, suggesting a potential for effectively using below-labeled herbicide rates (Johnson et al., 1997; Wait et al., 1999). In other cases, the use of reduced rates is only possible (efficient) when combined with other non-chemical practices such as cultivation and timing (Zhang et al., 2000), denoting the ability of weeds to withstand low herbicide rates.

Even though intentional or unintentional exposure to sublethal herbicide doses is a common event in target weed populations, the complete lack of critical experiments leads to a very little knowledge about the phenotypic responses associated with selecting target plants under recurrent reduced rates of herbicides.
Based on the fact that the widespread use of herbicides in agriculture makes highly probable the employment of recurrent applications (even with the same herbicide) within a growing season, the existence of anthropically- and environmentally-induced events that may reduce the effective recommended herbicide dosages in field conditions, and the innate capacity of plants to tolerate sublethal dosages of herbicide, a set of independent experiments were designed according to our goals: (1) to assess the effect of recurrently exposing single plants to sublethal rates of herbicide, (2) determine the capacity of plants selected with low rates to withstand higher concentrations of herbicide and (3) establish whether plant survival under continuously selection with low rates of herbicide is an inheritable phenotypic trait.

We believe that the answer to these questions will be of practical importance not only for the management and control of weeds but also in the context of comprehension of plant responses under sublethal stressful conditions.

As a model of study, we have concentrated on the effects of a potent lipid biosynthesis inhibitor and oxidative stress promoter herbicide (diclofop-methyl, DM, (methyl 2-[4-(2,4-dichlorophenoxy) phenoxy] propanoate)) (Gronwald et al., 1992; De Prado et al., 1999) on a fast-growing winter annual species (Lolium multiflorum Lam.).

2. Materials and methods

2.1. Plant material

A commercial (Oregon cv.), fungal endophyte-free and known DM-susceptible L. multiflorum population from Oregon (USA) was used. The extent of DM susceptibility was assessed by submitting the population to a range of DM rates (10, 100, 250, 500, and 750 μM, 1 and 50 mM) and compared to a well-known L. multiflorum resistant population developed by USDA-ARS National Forage Seed Production Research Center (ORARHR-G93, Gulf cv.) (Barker et al., 1997). DM was applied according to the methodology described in Vila-Aiub et al. (2003b).

We examined L. multiflorum seeds (n = 100) with a light microscope to estimate the incidence of Neotyphodium endophyte infection in the population (Latch et al., 1987).

Commercially formulated DM (EC 284 g a.i. L⁻¹) was provided by Hoechst-Aventis (Argentina). The commercial label dosage recommended for the formulated compound in Argentina is 2 L ha⁻¹ (560 g a.i. ha⁻¹) diluted in 100 L water ha⁻¹. All trials were carried out in the experimental campus of the Faculty of Agronomy, University of Buenos Aires (34°35'S, 58°35'W), under laboratory and field conditions during 1997–2001.

2.2. Exposure of individuals to recurrent sublethal rates

The experiment was conducted in a controlled environmental laboratory equipped with a horizontal laminar airflow workstation under conditions of fluctuating temperature (20±2°C), 10 h of daylight comprising the light phase coinciding the warm period and 100 μmol photons m⁻² s⁻¹ (photosynthetic active radiation, PAR) provided by fluorescent white light tubes.

Twenty seeds were surface sterilised (sodium hypochlorite 20%, v/v) for 15 min and germinated on filter paper moistened with distilled water on 6 mm deep and 9 cm-diameter circular Petri dishes. Four seedlings (genotypes 1, 2, 4 and 5) were chosen at random and transplanted into glass tubes (150 mm height and 20 mm diameter) containing 10 ml of a 0.7% (w/v) agar-water culture media: sucrose (30,000 mg L⁻¹), inositol (100 mg L⁻¹), NO₃-NH₄ (650 mg L⁻¹), NO₃-K (1900 mg L⁻¹), CaCl₂ (440 mg L⁻¹), SO₄-Mg (570 mg L⁻¹), KH₂PO₄ (170 mg L⁻¹), H₂BO₃ (6.2 mg L⁻¹), FeSO₄ (27.8 mg L⁻¹), MgSO₄ (37.2 mg L⁻¹), MnSO₄ (15.1 mg L⁻¹), ZnSO₄ (0.83 mg L⁻¹), MoO₄ (0.025 mg L⁻¹), CuSO₄ (0.025 mg L⁻¹), EDTA (37.2 mg L⁻¹), FeSO₄ (27.8 mg L⁻¹), benzyladenine (0.2 mg L⁻¹), indole-3-acetic acid (IAA) (1 mg L⁻¹), glycine (2 mg L⁻¹), thiamine (0.4 mg L⁻¹) and ampicillin (50 mg L⁻¹). When necessary the pH of the culture media was adjusted to 6 with KOH or NaOH before autoclaving it at 121°C for 20 min. Glass tubes were covered using a plastic transparent cap (Sigma, USA) immediately after transplanting the plants.

Each L. multiflorum genotype was asexually propagated until a total of approximately 500 homogeneous tillers (5 tillers per tube) were obtained. A prelimi-
nary experiment carried out with all the genotypes under the same environmental conditions as described above, indicated that, whereas, no survival was observed under exposure to the recommended DM rate (560 g a.i. ha$^{-1}$), between 35 and 65% of individuals survived when exposed to 16 and 8 times less rates. Solutions with a final DM concentration of 2.05 and 1.025 mM (herbicide acclimation rates of 35 and 70 g a.i. ha$^{-1}$, respectively) were prepared to treat the foliage of individual tillers with a soft painting brush. The same brush moistened with distilled water was used for the clones under no herbicide exposure.

The level of survival of each genotype to herbicide acclimation was estimated by assessing the capacity of herbicide treated tillers to produce new vegetative shoots (i.e. a herbicide susceptible tiller is not capable of producing new tillers). Three days after each herbicide application, 100 herbicide treated tillers were chosen at random and transplanted into a new media culture. Owing to the technique regarding vegetative propagation not being 100% efficient, the survival of each genotype to DM was affected by a correction factor:

$$\text{Survival (\%)} = \frac{S_t}{HS_t} \times 100 = \frac{S_t}{HS_t} \left( \frac{P_t}{PR_t} \right) \times HS_t \times 100$$

where $S_t$ is the number of surviving tillers, $HS_t$ the number of herbicide treated tillers, $C_f$ the correction factor which is determined by the number of produced tillers ($P_t$) in relation to the number of propagated tillers ($PR_t$) without herbicide exposure multiplied by $HS_t$.

We performed four acclimation cycles with sublethal dosages of DM. After each cycle, the surviving clones were asexually propagated until obtaining a new population of 500 tillers, and then subsequently exposed again with the same sublethal rate of DM (Fig. 1).

All data were analyzed by using the SAS Version 6.12 statistical package (SAS Institute, Cary, NC). As plant survival was measured on the same experimental units repeated over time, we performed a two-way analysis of variance (ANOVA) for repeated measures being the herbicide acclimation cycles ($AC$) and the rate of herbicide ($H$) as the main factors of the analysis. To comply with the assumptions of normal distribution and homoscedasticity, the values of plant survival were angular transformed (arcsine $\sqrt{x}$).

Plant survival means were separated using Tukey’s HSD multiple range test ($\alpha = 5\%$). Results are presented as mean (all genotypes) ± standard error of the mean.

2.3. Evaluation of plant herbicide dose tolerance after herbicide acclimation

After the fourth herbicide acclimation cycle, the plant material from each of the 12 treatments (genotype × sublethal rate) was asexually propagated, transplanted into plastic pots (20 cm upper diameter, 15 cm lower diameter, 20 cm height) and grown under...
2.4. CE experiment

Four hundred homogeneous tillers (approx. 33 tillers/pot) from each genotype × sublethal dose (35 and 70 g a.i. ha\(^{-1}\)) combination obtained at the controlled environmental laboratory were placed in a climatic growth chamber (ISCO: Instrumentation Specialties Company, NE, USA) with constant temperature (20°C), 10 h of daylight with a PAR intensity of 300 μmol photons m\(^{-2}\) s\(^{-1}\). Using three replicates per treatment, plant survival (%) was estimated 15 days since herbicide selection. New tillers produced by surviving plants were not considered when estimating plant survival. Before this experiment was carried out, the plant material from genotype 1 previously selected with 70 g a.i. ha\(^{-1}\) was lost due to fungus infection.

2.5. F experiment

Two hundred homogeneous tillers (10 per pot) were placed in an experimental garden during the normal growing season of _L. multiflorum_. Using five replicates per treatment, plant survival (%) and dry weight of surviving tillers were evaluated 30 days after herbicide selection. New tillers produced by surviving plants were not considered when estimating plant survival. Plant material was oven-dried for 3 days at 70°C before weighing.

Because a significant number of tillers of genotype 5 acclimated previously at 0, 35 and 70 g a.i. ha\(^{-1}\) of DM were infected by fungus before this experiment was carried out, the mentioned genotype was not considered.

2.6. Response to selection with sublethal herbicide rates

A significant number of tillers asexually propagated at the controlled environment laboratory were transplanted into plastic pots (20 cm upper diameter, 15 cm lower diameter, 20 cm height) containing a substrate with organic soil and sand (3:1, v/v) during the normal growing season of _L. multiflorum_. Given that _L. multiflorum_ is a cross-pollinated species, genotypes that had been selected with the same herbicide acclimation rates were isolated and allowed to cross pollinate and set seeds. A pollen proof barrier (frost protection net) was used during the flowering period to avoid pollen contamination among different treatments. At the end of the growing season mature seeds originated from each genotype cross were collected and stored in paper bags at lab temperature (~20°C).

The following growing season, 35 seeds from each of all genotype crosses that had been acclimatized at 0 (control), 35 and 70 g a.i. ha\(^{-1}\) of DM, were sown in plastic pots (20 cm upper diameter, 15 cm lower diameter, 20 cm height). Plants were sprayed with the following DM rates when they reached two-leaf stage: 0 (control), 70, 140, 280 and 560 g a.i. ha\(^{-1}\). Depending on seed availability, between three and four replicates were used per treatment. Plant survival (%) was evaluated 30 days after herbicide selection.

The main effects of genotype cross, herbicide acclimation, screening rate and their interactions were studied performing a three-way ANOVA (General Lin-
ear Model Procedure, SAS Version 6.12 statistical package, SAS Institute, Cary, NC). Survival was arcsine √x-transformed and additional comparisons of means were done using Tukey’s HSD multiple range test. The probability level of $P < 0.05$ was used to delineate significant main and interaction treatment differences. Results are presented as mean ± standard error of the mean.

3. Results

The *L. multiflorum* population (Oregon cv.) used in our experiments was highly susceptible to commercial rates (16 mM) of diclofop-methyl (Fig. 2). A detailed examination of its seeds also showed the complete lack of association with symbiotic fungal endophytes, which might have led to confounded effects on the observed responses.

3.1. Herbicide acclimation by recurrent exposure to sublethal rates

Herbicide dose and number of exposures to herbicide interacted ($P < 0.0001$) (Table 1), resulting in a progressive increase in the level of herbicide resistance in the tested *L. multiflorum* genotypes conferred by each of the first three consecutive diclofop-methyl applications (Fig. 3). Genotypes exhibited about 20% survival after the first exposure to 35 and 70 g a.i. ha$^{-1}$. However, when the surviving tillers comprising each genotype were exposed to the second and third diclofop-methyl application, survival increased to 70 and 80%, respectively. The survival attained after the fourth exposure to diclofop-methyl was similar to the third cycle (80%) (Fig. 3).

3.2. Evaluation of plant herbicide dose tolerance

Given a significant triple interaction, plant survival of *L. multiflorum* under controlled environmental conditions was affected not only by the level of exposure to herbicide screening rates (140, 280 and 560 g a.i. ha$^{-1}$) of diclofop-methyl, but also by the identity of the genotype and herbicide acclimation rates (35 and 70 g a.i. ha$^{-1}$) (Table 2).

Plants acclimated with 35 and 70 g a.i. ha$^{-1}$ rates displayed a greater survival than herbicide untreated individuals when exposed to higher diclofop-methyl rates (Fig. 4). Herbicide-acclimated plants of genotypes 2 and 4 survived between two-four fold at

![Fig. 2. Compared responses of known susceptible (■, Oregon cv.) and resistant (▲, Gulf cv.) *L. multiflorum* populations to varying dosage rates of diclofop-methyl. Points are mean survival of 20 seedlings and vertical bars are standard error (S.E.) of the mean ($n = 3$).](image_url)
Fig. 3. Survival of *L. multiflorum* genotypes to continuous exposure to sublethal rates of diclofop-methyl herbicide under environmentally controlled conditions. Symbols represent the mean survival of all genotypes and vertical bars denote S.E. of the mean (*n* = 4). Different letters indicate significant differences (*P* < 0.05) between survival values within each herbicide selection cycle.

140 and 280 g a.i. ha\(^{-1}\) rates of diclofop-methyl over herbicide-unselected plants. All genotypes (except genotype 4) showed no significant difference in survival between plants acclimated at 0, 35 and 70 g a.i. ha\(^{-1}\) when exposed to the highest diclofop-methyl rate (560 g a.i. ha\(^{-1}\)). Clones of genotype 1 acclimated at 35 g a.i. ha\(^{-1}\) only showed higher survival than clones from unselected individuals when exposed to 140 g a.i. ha\(^{-1}\). No difference in survival was observed between herbicide-acclimated and non-acclimated individuals from genotype 5 when exposed to herbicide screening rates (Fig. 4).

The analysis of variance for the experiment conducted at field conditions revealed that plant survival of *L. multiflorum* exposed to 140, 280 and 560 g a.i. ha\(^{-1}\) rates (herbicide screening rates) of diclofop-methyl depended on the identity of the genotype and herbicide acclimation rates (35 and 70 g a.i. ha\(^{-1}\)) (Table 2). A significant increase in survival was observed in plants herbicide acclimated with 35 and 70 g a.i. ha\(^{-1}\) rates and exposed to 140 and 280 g a.i. ha\(^{-1}\), compared to plants never acclimated with diclofop-methyl (Fig. 5). No difference in survival between herbicide-acclimated and non-acclimated plants was observed when exposed to field rates of 560 g a.i. ha\(^{-1}\). Depending on the rate of herbicide screening, herbicide-acclimated plants at 35 g a.i. ha\(^{-1}\) accumulated more aboveground biomass.

The analysis of variance revealed that the current herbicide environment (herbicide screening rates), genotype and herbicide exposure history (herbicide acclimation rate) had significant effects on plant survival and biomass of *L. multiflorum*.

Table 2

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Controlled environment experiment</th>
<th>Field experiment</th>
<th>Field experiment (F1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival</td>
<td>Survival</td>
<td>Biomass</td>
</tr>
<tr>
<td>Genotype (G)</td>
<td>0.0001</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>Herbicide acclimation rate (HAR)</td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.008</td>
</tr>
<tr>
<td>Herbicide screening rate (HSCR)</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G × HAR</td>
<td>0.001</td>
<td>0.38</td>
<td>0.003</td>
</tr>
<tr>
<td>G × HSCR</td>
<td>0.06</td>
<td>0.001</td>
<td>0.008</td>
</tr>
<tr>
<td>HAR × HSCR</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>G × HAR × HSCR</td>
<td>0.0004</td>
<td>0.56</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Analyses of data from three experiments carried out in environment controlled and field conditions. The experimental variables were estimated in genotypes during their vegetative growth and after sexual reproduction (progeny F1).

* For the evaluation of the progeny (F1) "genotype" denotes the identity of *L. multiflorum* genotype crosses.
Fig. 4. Survival of *L. multiflorum* genotypes acclimated with sublethal rates of diclofop-methyl (0, 35 and 70 g a.i. ha\(^{-1}\)) and exposed to herbicide screening rates of diclofop-methyl (0, 140, 280 and 560 g a.i. ha\(^{-1}\)) under environmentally controlled conditions. Vertical bars represent the mean survival and vertical lines denote S.E. of the mean (\(n = 3\)). Different letters indicate significant differences (\(P < 0.05\)) between survival values within each herbicide screening rate.

Fig. 5. Survival of *L. multiflorum* plants acclimated with sublethal rates of diclofop-methyl (0, 35 and 70 g a.i. ha\(^{-1}\)) and exposed to herbicide screening rates of diclofop-methyl (0, 140, 280 and 560 g a.i. ha\(^{-1}\)) under field conditions. Vertical bars represent the mean survival of all genotypes and vertical lines denote S.E. of the mean (\(n = 10–15\)). Different letters indicate significant differences (\(P < 0.05\)) between survival values within each herbicide screening rate.

Fig. 6. Survival of *L. multiflorum* plants after exposure to sublethal rates of diclofop-methyl (0, 35 and 70 g a.i. ha\(^{-1}\)) and exposed to herbicide screening rates of diclofop-methyl (0, 140, 280 and 560 g a.i. ha\(^{-1}\)) under field conditions. Vertical bars represent the mean survival of all genotypes and vertical lines denote S.E. of the mean (\(n = 10–15\)). Different letters indicate significant differences (\(P < 0.05\)) between survival values within each herbicide screening rate.

Regardless of the genotype and the environmental condition (growth chamber or field), individuals acclimated with 35 and 70 g a.i. ha\(^{-1}\) rates of diclofop-methyl showed consistently remarkable higher LD50 values than herbicide-unselected individuals (Table 3).

Table 3

<table>
<thead>
<tr>
<th>History of herbicide exposure (g a.i. ha(^{-1}))</th>
<th>Controlled environment experiment</th>
<th>Field experiment</th>
<th>Field experiment (F1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>24</td>
<td>225</td>
<td>96</td>
</tr>
<tr>
<td>35</td>
<td>507 (25)</td>
<td>700 (3)</td>
<td>49</td>
</tr>
<tr>
<td>70</td>
<td>2255 (94)</td>
<td>4290 (190)</td>
<td>63</td>
</tr>
</tbody>
</table>

The values were calculated from plants growing under controlled and field conditions during their vegetative growth and after sexual reproduction (F1). Values in parenthesis correspond to the resistance index calculated as the ratio between LD50 values of plants exposed to sublethal rates of herbicide and the control treatment.
Fig. 6. Aboveground biomass of surviving *L. multiflorum* plants acclimated with sublethal rates of diclofop-methyl (0, 35 and 70 g a.i. ha$^{-1}$) and exposed to herbicide screening rates of diclofop-methyl (0, 140, 280 and 560 g a.i. ha$^{-1}$) under field conditions. Vertical bars represent the mean survival and vertical lines denote S.E. of the mean ($n=5$). Different letters indicate significant differences ($P<0.05$) between survival values within each herbicide screening rate.
Table 4

Herbicide LD$_{50}$ values estimated in the progeny of different *L. multiflorum* genotype crosses after acclimation with sublethal diclofop-methyl rates and exposed to higher rates

<table>
<thead>
<tr>
<th>History of herbicide exposure (g a.i. ha$^{-1}$)</th>
<th>LD$_{50}$ (g a.i. ha$^{-1}$) cross between genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × 2</td>
</tr>
<tr>
<td>0 (control)</td>
<td>76</td>
</tr>
<tr>
<td>35</td>
<td>57.5</td>
</tr>
<tr>
<td>70</td>
<td>71</td>
</tr>
</tbody>
</table>

$^*$ Significant differences between LD$_{50}$ values within each plant cross.

Fig. 7. Survival of the progeny (F1) of *L. multiflorum* plants submitted to herbicide screening rates of diclofop-methyl (0, 70, 140, 280 and 560 g a.i. ha$^{-1}$) under field conditions. Parental lines were exposed to sublethal diclofop-methyl rates of 0, 35 and 70 g a.i. ha$^{-1}$. Vertical bars represent the mean survival of individuals from all genotype crosses and vertical lines denote S.E. of the mean (n = 10–15). Different letters indicate significant differences (P < 0.05) between survival values within each herbicide screening rate.

3.3. Response to selection with sublethal herbicide rates

Contrary to the experiments during the vegetative stage, there was an absence of significant interaction between the herbicide acclimation and screening rates on survival of F1 (Table 2). Based on this fact and on the evidence that progeny from all plant crosses possessed a similar response when exposed to herbicide selection (Table 4), the progeny of herbicide-acclimated and non-acclimated *L. multiflorum* showed the same levels of survival when exposed to screening rates (Fig. 7), leading to similar LD$_{50}$ values (Table 3).

Unexpectedly the cross between genotypes 1 and 5 from the herbicide untreated control had a significantly higher LD$_{50}$ value than the rest of the treatments (Table 4).

4. Discussion

The results of this study demonstrate that the level of herbicide resistance in *L. multiflorum* plants periodically exposed to sublethal dosages of herbicide (DM) dramatically increases. Twenty percent of the tillers of "susceptible" plants when exposed to 8 and 16 times less herbicide than that recommended by the manufacturer had the capacity to withstand the herbicide effects. However, only three consecutive application events with the same doses were required to build plants remarkably resistant (90%) to diclofop-methyl herbicide, suggesting an acclimation response (Fig. 3). This revealed the existence of variation between propagules of single plants (somatic variation) associated with the tolerance to DM. Somatic variation has been frequently found in grass species as accounted, for example, in previous reports, which documented the existence of this type of variation in the closely related *L. perenne* for tillering rate and plant height (Breese et al., 1965; Shimamoto and Hayward, 1975).

Sublethal herbicide-acclimated single plants were able to tolerate later herbicide selections using four times higher rates (Fig. 4). Plants acclimated with 35 and 70 g a.i. ha$^{-1}$ showed significantly higher survival and vegetative biomass when exposed at 140 and 280 g a.i. ha$^{-1}$ than herbicide-non-acclimated plants in environmental controlled and field conditions. Note-worthy, the differences we found between clones in the
sublethal-herbicide acclimation responses denotes the existence of intraspecific variation at the population level. Whereas, genotype (1) only when acclimated with the lowest diclofop-methyl rate (35 g a.i. ha$^{-1}$) and subsequently exposed to 140 g a.i. ha$^{-1}$ differed in survival and biomass in relation to herbicide non-selected plants, genotype (4) was the only one to display a higher survival at 560 g a.i. ha$^{-1}$ (commercial rate) when selected with the highest diclofop-methyl acclimation rate (70 g a.i. ha$^{-1}$).

Confirmation of acclimation as the event that took place during the increase of tolerance in the plants treated with sublethal diclofop-methyl rates was uncovered after examining that progeny of herbicide pre-treated L. multiflorum clones experienced similar rates of survival to herbicide selection compared to the progeny generated by non-treated clones (Fig. 7).

The results of our study, which may be the first experimentally documented evidence of induced herbicide tolerance (acclimation) in vascular plants, are consistent with the acclimation events observed among plants under sublethal stress conditions such as heavy metals (Cu, Zn, Cd), salt (NaCl) and ozone (O$_3$) (Cox and Hutchinson, 1980; Brown and Martin, 1981; Amzallag et al., 1990; Held et al., 1991; Ostridge and Hutchinson, 1991). Tolerance to cadmium (Cd) concentrations of 1 µg m$^{-1}$ was triggered by root pre-exposure at 0.2 µg m$^{-1}$ in a Cd-susceptible Holcus lanatus population (Brown and Martin, 1981). A similar response has been observed in Sorghum bicolor (L.) Moench when exposed to non-lethal conditions of salt (NaCl) (Amzallag et al., 1990). Treated plants with 75 or 150 mol m$^{-3}$ for 20 days became capable of tolerating concentrations of 300 mol m$^{-3}$ compared to untreated plants or plants that were pre-treated for less than 20 days.

Diclofop-methyl phytotoxicity in annual Loliurn species has been consistently linked to the inhibition of a key enzyme involved in lipid biosynthesis (ACCase, acetyl-CoA carboxylase) (Gronwald et al., 1992; Tardiff and Powles, 1994). However, a resistance mechanism related to the maintenance of transmembrane electrochemical gradients under diclofop-methyl selection has also been reported in some populations possessing a insensitive ACCase enzyme (Hauser et al., 1991). The herbicide resistance profile of the L. multiflorum population tested in our study (Oregon cv.) has revealed the absence of an insensitive ACCase enzyme (Fig. 2). Then, a recent report on the same population has shown that within 10 h after diclofop-methyl exposure, an increased activity of plasmatic membranes (H$^+$ extrusion) under sublethal diclofop-methyl rates accounted for the greater survival (15%) compared to that observed at higher diclofop-methyl rates (0-5%) (Vila-Aiub et al., 2003b). Given that plant acclimation is a phenotypic response that implies the ability to adjust physiological and structural attributes on the scale of seconds or seasons within a single genotype (Orcutt and Nilsen, 2000), we may well consider that the mentioned increased activity in cell membranes is a generalized response of L. multiflorum plants (Oregon cv.) that either operates or is induced under low diclofop-methyl rates.

Acclimation to stresses leading oxidative damage such as light, low temperature, drought and pollutants is also a common response among plants (Smirnoff, 1995). Due to diclofop-methyl has also shown to promote oxidative stress in Loliurn species (De Prado et al., 1999), the induction of an elevated expression of detoxifying or antioxidant enzymes as a successful mechanism under sublethal herbicide concentrations should not be laid aside.

Attempts to increase the efficacy of weed control have lead to research efforts on the use of reduced rates. An analysis of the general trend has shown that there is a high potential of reducing rates for preemergence and preplant incorporated herbicides when combined with interrow cultivation, denoting the importance of keeping strong selection pressures on weeds (Zhang et al., 2000). However, even with cultivation or different application timings unsatisfactory weed control may be achieved using reduced rates of postemergence herbicides such as diclofop-methyl (Spandl et al., 1997; Stougard et al., 1997; Zhang et al., 2000).

Based on results from studies using reduced rates of herbicides and on the fact that the chances of receiving sublethal herbicide rates are more likely to occur in weed species with strategies that involve perennial growth habits, then having a greater potential to develop the acclimation responses observed in this research compared to annuals, any recommendation about the sustainable use of herbicide reduced rates to control weeds, should acknowledge (a) the existence...
of intraclonal and intraspecific variability associated with herbicide resistance in weed populations when exposed at low rates, (b) the potential risk of exposing weed populations with reduced rates given the latent acclimation events that may occur under these herbicide conditions, as demonstrated in this study, and (c) the generalizations about the use of reduced herbicide rates to control weed populations should only be made from experimental results obtained in long-term studies.

In conclusion, a remarkably increase in herbicide tolerance of “daughter” ramets resulting from prior acclimation to sublethal rates by “parent” plants is possible in an annual weed species (L. multiflorum). Further research is required to elucidate whether the induction or acclimation to herbicide tolerance via sublethal rate exposure is a widespread phenomenon in response to different herbicide classes and among weeds with perennial growth habits.

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References


