

Evolution, growth and phenology of *Phalaris minor* biotypes resistant to ACCase-inhibiting herbicides in Mexico



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Abstract

Background: Herbicide application to control weeds can promote a rapid selection of resistant phenotypes in small geographic areas. Also, in areas with a spatial heterogeneous management, resistance may evolve independently, promoting local adaptation in weeds. In the Mexican region known as “El Bajío,” 100,000 ha are cultivated with wheat, and the weed *Phalaris minor*, resistant to ACCase-inhibiting herbicides is commonly present.

Question: We aim to identify the population structure of two genes in four different *P. minor* biotypes from “El Bajío” and to determine their association with phenology and plant growth differences (biomass and seed yield) that may contribute to survival in the agricultural environment.

Studied species: *Phalaris minor* Retz.

Study site and years of study: The study was carried out at a greenhouse and at the molecular biology laboratory of the Colegio de Postgraduados, during 2013 and 2014.

Methods: The diversity of the *psbA* gene and the sequence of two ACCase gene fragments as well as phenology, growth and biomass allocation were evaluated.

Results: Results indicated different polymorphism levels for the two genes. There were no differences in the *psbA* gene between biotypes, although the ACCase gene exhibited high polymorphism level. In addition, each biotype showed differences in phenology, biomass accumulation and fecundity.

Conclusions: The ACCase-inhibiting herbicide resistance in “El Bajío” region might be a resistance hot-spot leading to the local adaptation of weeds.

Key words: Evolution, herbicide resistance, local adaptation.

Resumen

Antecedentes: La aplicación de herbicidas para el control de las malezas puede fomentar una rápida selección de fenotipos resistentes en áreas geográficas pequeñas. También, en áreas con un manejo heterogéneo espacial, la resistencia puede evolucionar independientemente, promoviendo adaptaciones locales de las malezas. En la región mexicana conocida como “El Bajío”, se cultivan 100,000 ha con trigo, y la maleza *Phalaris minor*, resistente a herbicidas inhibidores de la ACCasa está comúnmente presente.

Pregunta: El objetivo fue identificar la estructura poblacional de dos genes en cuatro biotipos diferentes de *P. minor* de “El Bajío” y determinar su asociación con las diferencias en fenología y crecimiento de la planta (biomasa y rendimiento de semilla) que puedan contribuir a la sobrevivencia en campos agrícolas.

Especie estudiada: *Phalaris minor* Retz.

Sitio y años de estudio: El estudio se realizó en un invernadero y en el laboratorio de Biología Molecular del Colegio de Postgraduados durante 2013 y 2014.

Métodos: La diversidad del gen *psbA* y la secuencia de dos fragmentos del gen ACCasa fueron evaluados, así como también la fenología, el crecimiento y la asignación de biomasa.

Resultados: Los resultados indicaron diferentes niveles de polimorfismo para los dos genes. No hubieron diferencias en el gen *psbA* entre los biotipos, aunque el gen ACCasa exhibió nivel alto de polimorfismo. Además, cada biotipo mostró diferencias en la fenología, acumulación de biomasa y fecundidad.

Conclusiones: La resistencia a herbicidas inhibidores de la ACCasa en la región de “El Bajío” puede ser debida a parches que conducen a adaptaciones locales de las malezas.

Palabras clave: Adaptación local, evolución, resistencia a herbicidas.

The application of systemic herbicides to control weeds exerts intense selection pressure on weed populations (up to 99.99 %) (Maxwell *et al.* 1990), and the extensive application of herbicides with the same mode of action selects for mutations at the site of action or changes plant metabolism that allows plants to resist these herbicides (Powles & Yu 2010). The intense selection pressure reduces the effective plant population size to only few resistant individuals. The continued use of the same herbicide on each generation increases the number of resistant individuals that can outcompete susceptible individuals (Maxwell *et al.* 1990).

Weed populations in neighboring geographic areas can evolve relatively quickly to herbicide resistance (Baucom & Holt 2009), because crop management depends on farmer decisions, like timing, doses, and the number of applications as well as the kind of herbicide applied (Délye *et al.* 2004, Owen *et al.* 2011), herbicide resistance might occur independently among cultivars. For example, in localities in close spatial proximity, such as in the case of *Alopecurus myosuroides* Huds. in Europe (Délye *et al.* 2004, Délye *et al.* 2010). Further, the cultural practices that include the sharing of equipment and seeds, along with seed and pollen dispersal, enable resistant biotypes to disperse over large areas (Afentouli & Eleftherohorinos 1996).

Such scenario is likely in the central region of Mexico known as “El Bajío” (20° 25’ N 101° 38’ W), where ca. 100,000 ha are cultivated with wheat although individual farmers cultivate parcels of ground 1-2 ha. Here, *Phalaris minor* Retz. is the main problem in wheat cultivation. *P. minor* (Poaceae) is an Asian weed dispersed worldwide. This weed is considered as one of the worst weed for wheat production (Afentouli & Eleftherohorinos 1996). This plant has an erect habit with stems up to 180 cm tall, its leaves are long, linear and acuminate. The inflorescences are panicles that produces a high amount of seeds. The principal way of dispersion is through the seed. The seeds of *P. minor* is around 2 mm of length and 1.7 mg of weight. Each plant can produce around 5,000 or even more seeds, depending the number of spikelets produced (Shukla 1996).

Since 1996, biotypes resistant to ACCase-inhibiting herbicides have been reported on at least 100 farms, affecting approximately 5,000 ha (Heap 2016). Using dose response tests, the presence of resistant *P. minor* populations has been documented in at least four sites (Tafuya & Morgado 2000).

Because of the geographic distance between sites and differences in crop management, each site might constitute a hot spot of herbicide resistance evolution. Furthermore, each resistant biotype might be locally adapted (life cycle and physiology) as a result of selection imposed by agricultural practices (Weinig 2005). For the biotypes present in this zone, some studies have shown differences in the early stages of plants life history, including changes in seed physiology (germination rate, dormancy and longevity) and growth for canopy competition (Torres-García *et al.* 2015a, 2015b). In addition, empirical evidence based on morphology suggests differentiation among resistant weed populations (García-Franco *et al.* 2014). To date, the molecular basis of herbicide resistance and the biological characteristics of *P. minor* populations at “El Bajío” have not been yet described.

We hypothesize that *P. minor* biotypes encode different polymorphisms in the gene that confers resistance to herbicides (ACCase) as well as in a non-related gene (*psbA*). Furthermore, the biotypes should exhibit differences in phenology, growth rates, and reproductive output derived from farmers’ management practices. In this study, we aim to determine 1) if the genes *psbA* and ACCase from *P. minor* biotypes from the “El Bajío” show polymorphisms associated with herbicide resistance selection, and 2) to what extent it leads to differences in phenology and growth (biomass and seed yield) that allow survival in the agricultural environment.

Materials and methods

A collection of at least 20 populations of *P. minor* reported to be resistant (R) to clodinafop-propargyl was generated in “El Bajío,” Mexico (20° 25’ N 101° 38’ W). One biotype from a location 85 km further apart, where the plants grew outside crops never subjected to chemical control (personal communication of farmers) was used as the susceptible line (Figure 1). Dose-response resistance were performed for each population. Potential resistance of each biotype was determined in Petri dishes with agar gel containing clodinafop-propargyl (20 mL L⁻¹) (Tal *et al.* 2000). The four biotypes with the highest herbicide resistance were selected: Col 4, Col 7,

Author contributions

Jesús Rubén Torres García designed and set up the experiment, evaluated and analyzed data and wrote the paper.

Obdulia Segura León helped to carried out molecular analysis.

Ebandro Uscanga Mortera designed the experiment and help to write the paper.

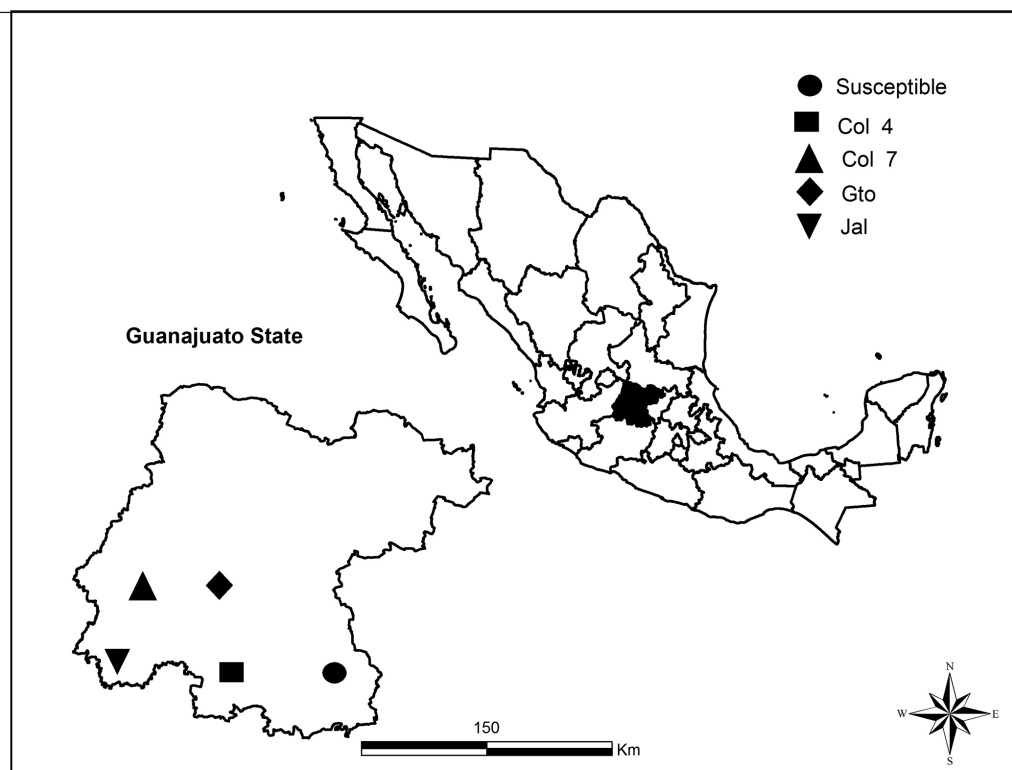
Carlos Trejo determined the physiological variables to take and make the interpretation.

Víctor Conde Martínez helped to write the paper.

Josué Kohashi Shibata make a critical reviewed and suggested changes on the paper.

David Martínez Moreno helped to analyze the data.

Figure 1. Geographic localities where biotypes of *Phalaris minor* were sampled in “El Bajío,” Mexico.



Gto and Jal. A dose response test was performed on these biotypes in a greenhouse to determine if these resist to field doses of herbicide application. The biotypes Col 4, Col 7 and Gto survived in doses 12-fold higher than the field dose, whereas the Jal biotype showed 6.8-fold resistance in relation to the susceptible line.

Seedlings of the selected biotypes were grown in pots. Twenty days after emergence, clodinafop-propargyl was applied at a rate of 120 g *a.i.* ha⁻¹ to each population. Plants that survived herbicide applications were maintained to produce seeds. Seeds were stored in laboratory conditions and used in all further experiments reported here.

Molecular basis of resistance. Seeds of different biotypes were germinated and grown until the appearance of the first leaves. DNA was extracted from 5 individuals from each population. DNA extraction was performed using the CTAB method (Stewart & Via 1993). To determine whether herbicide resistance in these biotypes was conferred by mutations at the site of action, two regions of the ACCase gene (from position 5,109 to 5,632 and 5,950 to 6,355) and *psbA* were amplified. Two sets of universal primers for ACCase that were developed by Délye & Michel (2005) were used for amplification (Table 1). The PCR mix contained 1X buffer, 10 mM dNTPs, 1 µL DNA (50 ng µL⁻¹), 1.5 mM MgCl₂, 1 U Taq (Flexitaq; Promega), and 10 mM each primer at a final volume of 25 µL. The amplification conditions were as follows: denaturation at 95 °C for 30 s; followed by 37 cycles of 95 °C for 10 s, 60 or 61 °C (depending on the fragment) for 15 s, and 72 °C for 45 s; followed by a final extension at 72 °C for 10 min. Gene amplification was performed in a Biometra thermocycler (TPersonal model).

For *psbA* amplification, a *P. minor* sequence reported in GenBank (AY211527; Tal & Rubin 2002, unpublished) was used for primer design. The primers were designed using Primer3 software (Untergasser *et al.* 2007) (Table 1). The PCR conditions were similar to those used for the ACCase gene, and the amplification program was as follows: 95 °C for 120 s; followed by 30 cycles at 95 °C for 60 s, 52 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 8 min. The PCR products were separated on 0.8 % agarose gels in 1 % TBA buffer at 76 V for 20 min. Positive amplicons were sequenced by Macrogen, Inc. (South Korea).

A sequence analysis was performed using MEGA (Molecular Evolutionary Genetic Analysis) software version 6 (Tamura *et al.* 2013). Prior to analysis, we confirmed the quality and

Table 1. Name, sequence, fragment size (bp) and annealing temperature (°C) of the primers used to amplify the ACCase gene (ACcp1, ACcp1R, ACcp4 and ACcp2R) (Delyé & Michel 2005).

Primer	Sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)
psbA F	CTACCTTATTGACTGCAACT	750	52
psbA R	TTAGGTTGAAAGCCATAGT		52
ACcp1	CAACTCTGGTGCTIGGATIGGCA	523	60
ACcp1R	GAACATAICTGAGCCACCTIAATATAT		60
ACcp4	CAGCITGATTCCCAIGAGCGITC	405	61
ACcp2R	CCATGCAITCTTIGAGITCCTCTGA		61

effective size of the sequences included in the alignment. The sequences were aligned with the program Clustal W, and translation to amino acids was performed using the chloroplast genetic code. Each amplified region was analyzed independently. For the ACCase gene, the two fragments were linked, indicating the start and end site of each fragment. The alignment of each gene included a *P. minor* sequence from Israel that is reported in GenBank as susceptible to ACCase-inhibiting herbicides (AY196481, AY211527).

Evaluation of growth. To assess possible differences among herbicide-resistant populations, 40 seeds of each resistant biotype were sown in pots (1.7 L capacity) filled with forest soil and sand in a 2:1 ratio. The moisture of the substrate was maintained near field capacity throughout the entire experiment, which was performed in two seasons: autumn-winter (October through March) and spring-summer (April through August). The minimum and maximum daily temperatures in the greenhouse were recorded during the experiment. The number of growing degree days (GDD) was calculated according to Forcella & Banken (1996): $GDD = [(T_{max} + T_{min}) / 2] - T_{base}$. The base temperature for *P. minor* was estimated to be 10 °C.

Phenology and accumulation of growing degree days. The phenological stages were recorded during the experiment. These stages included: emergence, the occurrence of the second and fourth leaf blades, the occurrence of the spike and the end of anthesis. It was assumed that each stage was achieved when 50 % of the plants in each biotype exhibited the above-mentioned characteristics. We estimated the number of GDD between the phenological stage and subsequent stages.

Production and biomass allocation. At each phenological stage, 10 plants were destructively sampled to obtain dry matter. These plants were dissected into different structures (roots, culms, leaf blades, inflorescences and seeds). The organs were placed in paper bags and dried to a constant weight at 80 °C. The weight (g) of each organ was recorded to obtain the total biomass weight per plant. The dry matter percentage allocation for each organ was also determined. Finally, we counted the number of culms, leaf blades, tillers and spikes in each plant.

Growth analysis. We used the methodology proposed by Hunt (1978) to evaluate the absolute growth rate (AGR), relative growth rate (RGR), net assimilation rate (NAR) and leaf area duration (LAD). The LAD is usually expressed in days; however, because of the growth conditions in the greenhouse, we expressed this calculation in $dm^2 d^{-1}$ (Hunt 1978).

Statistical analysis. For the statistical analysis each biotype was considered as a treatment. In the harvests 10 replicates per biotype were used. The second variation factor was the growing season (autumn-winter and spring-summer). A completely randomized design was used in both growing seasons. To remove the seasonal effect, autumn-winter and spring-summer data were analyzed as a series of experiments in time, in which the growing season is nested within replicates (Cochran & Cox 1990). A mixed model was used, the biotype was considered as a random factor and the season as fixed factor. Prior to statistical analyses, the percentages were transformed with the arcsine function, and after analyses, the data were transformed back to

their original scale. Significant differences between the treatment means were identified with Tukey's multiple comparison test ($P < 0.05$).

Results

Molecular basis of resistance. Results from sequencing ACCase and *psbA* gene fragments showed different variation patterns among biotypes. The gene *psbA* showed no variation; and in the ACCase gene, we found 24 variable sites out of 752 analyzed nucleotides. Within each biotype, different number of variable sites were observed, and the biotype with more variable sites was Col 4 (16 sites), followed by Gto (14 sites), Col 7 (13 sites) and Jal (8 sites) (Table 2).

Table 2. Mutations in the site of action in Littleseed canarygrass biotypes resistant to ACCase-inhibiting herbicides in "El Bajío," Mexico (Col 4, Col 7, Gto and Jal).

	Sec	Hap	sites	Variable sites	Substitutions
Susceptible	5	5	752	14	
Col 4	5	5	752	16	2041 Ile x Asn
Col 7	5	5	752	13	2078 Asp x Gly
Gto	4	4	752	14	1781 Ile x Leu
Jal	5	4	752	8	2027 Trp x Cys
Total	24	23	752	24	

Abbreviations: Hap = haplotypes.

Translation of the DNA sequence to the amino acid sequence showed that not all the changes modified the protein structure, as most mutations were synonymous. The amino acid sequence of the S line was identical to that of Israel sequence labeled as susceptible to ACCase-inhibiting herbicides in GenBank (AY196481). All biotypes showed only one amino acid change compared to S, and this change occurred at different positions in each biotype: Col 4 (2041 Ile x Asn), Col 7 (2078 Asp x Gly), Gto (1781 Ile x Leu) and Jal (2027 Trp x Cys).

Phenology and accumulation of growing degree days. The studied biotypes showed phenology differences during the experiment. The biotypes Col 7 and Jal exhibited a similar phenology and GDD accumulation, 163 d (3,265 GDD) and 159 d (3,222 GDD), respectively (Table 3). In contrast, the Col 4 and Gto biotypes showed early development. Differences in among biotypes were observed since the first harvest, and become more evident at the end of the experiment. Flowering time in both R and S biotypes occurred at 90 d (1,851 GDD).

Table 3. Days after germination and growing degree days (GDD) accumulated during the biomass sampling of Littleseed canarygrass biotypes resistant to ACCase-inhibiting herbicides in "El Bajío," Mexico.

Biotype	2nd leaf blade		4th leaf blade		Beginning of flowering		Physiological maturity	
	Days	GDD	Days	GDD	Days	GDD	Days	GDD
Col 4	15±3	306±28	26±4	522±41	90±4	1851±40	140±4	2892±38
Col 7	15±3	312±28	40±5	588±47	134±7	2720±67	162±6	3222±62
Gto	12±2	247±18	26±5	522±49	90±3	1851±32	143±7	2926±68
Jal	18±4	372±36	37±4	733±38	121±6	2502±55	159±5	3265±55

Production and distribution of biomass. In terms of biomass accumulation at physiological maturity, Jal showed the highest accumulation, followed by Col 7. In contrast, the biotypes with early growth (Col 4 and Gto) produced less biomass. Biomass allocation to different plant organs indicated that, in all of the biotypes, the highest fraction of biomass was allocated to roots (*e.g.*, 49.2 % in Col 7) followed by the culms, leaves and seeds. Biotypes with early growth showed a reduction in biomass allocation to roots of up to 20 % with respect to S biotype (Table 4).

With respect to the aerial biomass, Jal exhibited the greatest dry matter accumulation (culms, leaves and inflorescences). In contrast, Gto exhibited the lowest accumulation in these struc-

Table 4. Dry biomass allocated to roots, culms, leaf blades and total biomass (g plant⁻¹) in *Phalaris minor* biotypes resistant to ACCase-inhibiting herbicides in “El Bajío,” Mexico. Different letters indicate statistically significant differences.

	Root		Culm		Leaf blades		Spikes		Seeds		Total
	g	%	g	%	g	%	g	%	g	%	g
Col 4	8.2±1.3 b	38.5± 6.1b	6.0±2.2 b	28.2± 10.3ab	3.2±0.8 b	15.0±3.8 b	1.3±0.4 b	6.1±1.9 a	2.6±0.5 ab	12.2±2.3 a	21.3 c
Col 7	19.3±3.4 a	49.2±8.7 a	10.0±3.1 a	25.5±7.9 ab	7.2±1.2 a	18.4±3.1 ab	0.2±0.1 c	0.5±0.3 d	2.5±0.3 ab	6.4±0.8 b	39.2 b
Gto	5± b0.8	31.8±5.1 b	4.8± 1.7b	30.6±10.8 a	3.2±0.4 b	20.4±2.6 a	1.1±0.3 b	7.0±1.9 b	1.6±0.2 b	10.2±1.3 a	15.7 d
Jal	21.2±3.2 a	46.5±7.0 a	11.0±2.0 a	24.1±4.4 b	8.0±0.9 a	17.5±2 ab	2.4±0.5 a	5.3±1.1 a	3.0±0.4 ab	6.6±0.9 b	45.6 a
SIGNIFI- CANCE	***	***	***	***	***	***	***	***	**	***	***
HSD (Tukey test, 0.05)	4.4	7.8	3.2	6.2	1.6	3.5	0.7	2.4	1.5	3.6	5.5

*, ** and *** indicate $P < 0.5$, 0.01 and 0.001, respectively.

tures (Table 4). The differences between biotypes were related to the number of culms, and leaves in biotypes with a long life cycle which had the greatest number of leaves and, therefore, the greatest leaf area (Table 4).

No difference among the biotypes Col 4, Col 7 and Jal in seed production (g plant⁻¹) were observed. The Gto biotype showed the lowest seed production per plant. However, biomass allocation showed a different trend; the short-cycle biotypes allocated more biomass matter to seeds than the long-cycle biotypes (Table 2). The increase in biomass allocation to seeds in the short-cycle biotypes occurred because flowering began 30 d earlier; therefore, these plants had more time for seed formation. This increase might also be a compensatory response, *e.g.*, by decreasing the size of the plant, more dry matter can be allocated to seed production (reproductive effort).

Growth analysis. The growth rate exhibited a trend similar to that observed for biomass accumulation. The absolute and relative growth rates (AGR and RGR) were highest in the biotypes with the greatest biomass accumulation (Jal and Col 7) (Table 5). The RGR data showed that Jal and Col 7 had the highest efficiency with respect to biomass production from existing biomass. No difference in NAR was found between biotypes. The LAD was greater in Col 7 and Jal than in Col 4 and Gto, due to a higher biomass accumulation in leaves, a larger number of leaves and a greater leaf area.

Table 5. Growth analysis of *Phalaris minor* resistant to ACCase-inhibiting herbicides biotypes in “El Bajío,” Mexico. Absolute growth rate (AGR), relative growth rate (RGR), net assimilation rate (NAR) and leaf area duration (LAD). Different letters indicate statistically significant differences.

Biotypes	AGR	RGR	NAR	LAD
	g d ⁻¹	g g ⁻¹ d ⁻¹	g dm ⁻² d ⁻¹	dm ² d ⁻¹
Col 4	91.7±8.7 b	0.26±0.1 b	0.64±0.07 a	204±57 b
Col 7	158.6±8.6 a	0.29±0.1 a	0.57±0.08 a	576±25 a
Gto	82.7±6.9 b	0.27±0.1 b	0.50±0.1 a	278±62 b
Jal	169.6±11.2 a	0.28±0.1 ab	0.61±0.09 a	548±42 a
SIGNIFICANCE	***	***	***	***
HSD (Tukey test, 0.05)	12.86	0.0012	0.14	118

*, ** and *** indicate $P < 0.5$, 0.01 and 0.001, respectively.

Discussion

Results of this study showed different variation patterns: no variation was observed in the *psbA* gene, whereas the ACCase gene showed more variation, indicating a different evolution history (pressure) for these genes. The ACCase gene is associated with herbicide resistance, and the polymorphism in the analyzed biotypes is associated with the selection pressure on weed populations in the “El Bajío” region in the central part of Mexico. The similarity between the suscep-

tible individuals from this work and from Israel supports the idea that changes at the nucleotide and amino acid level in other biotypes is associated with resistance to herbicides. However, the nucleotide and amino acid mutations in different codons suggest that more than one selective event have occurred in the “El Bajío” region in the central part of Mexico. This might be associated with farmers’ practices for weed management. A similar result in the ACCase gene has been documented by Délye (2005). Further, all mutations found in samples from the “El Bajío” region have been reported previously by Kaundun (2014) in grasses. For example, in the Gto biotype, a mutation in position 1,781 (Ile x Leu) was found and it has been reported to confer resistance to three chemical families (aryloxyphenoxypropionate, cyclohexanedione and phenylpyrazoline) (Powles & Yu 2010). The use of chemical families of ACCase inhibitors, such as aryloxyphenoxypropionate and cyclohexanedione, is common in “El Bajío.”

The polymorphism found in the ACCase inhibitor in the studied area shows that selective pressure from different herbicides could be underway because herbicide application is based on farmer’s decision and this may have implications for weed management. The evolution of resistant populations in close geographic proximity possibly have evolved independently, as it has been observed in regions of Australia (Owen *et al.* 2011). Several studies have demonstrated the multiple genetic origins of herbicide resistance in crop areas; Délye *et al.* (2004, 2010) found that resistance to ACCase-inhibiting herbicides in populations of *Alopecurus myosuroides* in Europe (Germany, Belgium, France, Netherlands, United Kingdom and Turkey) had a different evolutionary origin because of different weed management programs in each country.

Moreover, changes in phenology, growth, biomass accumulation and fecundity were found among biotypes. Reductions in the biological cycle of the Gto and Col 4 biotypes were observed and might be related to selection pressure on life history components in these populations. Changes in phenology also have been observed as an evolutionary response to adapt to agroecosystems, mainly in relation to the timing of the crop cycle (Duke 1985, Weinig 2005, Kawecki 2008) and in response to the application of herbicides and other cultural practices (Mortimer 1997). These characteristics could be an indicator that the biotypes Col 4 and Gto, in addition of being resistant, are also adapted to agricultural practices. As suggested by Baker (1974), the reductions in life cycle and biomass represent adaptations to an ephemeral life cycle that ensures seed production. However, Grime (1979) indicated that weeds have evolved to adapt to sites with high productivity and disturbance, such as crop fields. These changes, along with control methods, such as crop rotation, changes in herbicide active ingredients and others, can change the dominance of resistant individuals in populations. Information regarding the life cycle of weeds can aid our understanding of population dynamics in the absence of the selection factor and the return of susceptibility in populations (Maxwell *et al.* 1990).

All of the results obtained from *P. minor* from “El Bajío” suggest the development of resistance, which could be the result of the use of more than one herbicide family, because more than one amino acid changed in a small geographic area; this also supports changes in phenology and biomass accumulation. The variation mosaic present in the zone suggests a diverse diversification pattern in resistance. Thus, it is important long term studies that help to understand the evolution of herbicides resistance in a context of population differentiation, variable management practices, gene flow, and landscape. This may help to develop strategies to reduce the use of herbicides (for instance, altering the application both temporally and spatially), and to increase yield.

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