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The development of sulfonylurea herbicide-resistant birdsfoot trefoil (*Lotus corniculatus*) plants from in vitro selection

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Summary. Herbicide-resistant lines of birdsfoot trefoil (*Lotus corniculatus* L. cv 'Leo') were isolated after sequential selection at the callus, shoot, and whole plant levels to the sulfonylurea (SU) herbicide Harmony {DPX-M6316; 3-[[[(4-methoxy-6methyl-1,3,5, triazine-2-yl) amino] carbonyl] amino] sulfonyl-2-thiophenecarboxylate}. In field and growth chamber tests the Harmony regenerant lines displayed an increased tolerance as compared to control plants from tissue culture and controls grown from seed. Results of evaluation of callus cultures of regenerated mutant lines signify stability of the resistance. Outcrossed seeds collected from field trials, and tested *in vitro* for herbicide resistance, indicate that the trait is heritable and that resistance may be due to reduced sensitivity of acetolactate synthase to SU inhibition. Genetically stable herbicide-resistant lines of birdsfoot trefoil were successfully isolated using *in vitro* selection.

Key words: *Lotus corniculatus* – Herbicide resistance – Sulfonylurea herbicide – In vitro selection – Acetolactate synthase

Introduction

Birdsfoot trefoil, *Lotus corniculatus* L. cv 'Leo,' a forage legume widely grown in the northeastern United States and Canada, has many unique qualities such as high tolerance to poor soil, long persistence, bloat safety, and a forage quality comparable to alfalfa (Grant and Marten 1985). However, slow seedling growth, the inability to compete with weeds, coupled with a sensitivity to herbicides have all contributed to problems in establishing large stands (Hoveland et al. 1982). Wakefield and Skaland (1965) reported that weed control measures substantially increased yield to trefoil compared to untreated stands. On the other hand, several pre- and postemergent broadleaf herbicides tested in trefoil stands cause severe yield reduction. A herbicide that is capable of controlling weeds and not adversely affecting trefoil would be of value. Unfortunately, due to the costs involved in research, testing, marketing, and registration, new herbicides are very expensive to produce. A solution to this problem would be the development of cultivars with genetic tolerance or resistance to already existing herbicides. However, to date selection of herbicide-resistant trefoil has been only marginally succesfull (Devine et al. 1975; Swanson and Tomes 1980a,b; MacLean and Grant 1987).

Sulfonylurea herbicides, the best known of which is chlorsulfuron (trade name Glean), were developed by Du Pont de Namours to control broadleaf weeds in cereals. Important features of these herbicides include low application rates, a short soil half-life, and low mammalian toxicity (Levitt 1983). Chlorsulfuron (CS) acts by binding to and inactivating the acetolactate synthase (ALS) enzyme, inhibiting the biosynthesis of the essential branched-chain amino acids (Ray 1984), and Harmony is also reported to act on ALS (Sionis et al. 1985). Genetically stable CS tolerant fertile plants have been isolated using cell culture techniques in tobacco (Chaleff and Ray 1984), *Brassica napus* (Swanson et al. 1988), and flax (Jordan and McHughen 1987).

The objectives of this study were to select for regenerants of *Lotus corniculatus* cv 'Leo' tolerant to the herbicide Harmony {DPX-M6316; 3[[[(4-methoxy-6methyl-1,3,5,triazine-2-yl) amino] carbonyl] amino] sulfonyl-2-thiophenecarboxylate} using *in vitro* tissue culture selection techniques, and to evaluate this resistance at the cellular, whole plant, and biochemical levels.

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Materials and methods

Plant material

Seeds of birdsfoot trefoil cv Leo were obtained from the Emile Lods Agronomy Research Station at the Macdonald Campus of McGill University. The seeds were scarified in liquid nitrogen to improve germination, after which all subsequent procedures were performed aseptically in a laminar air-flow hood.

Media preparation and callus initiation

The protocols followed for media formulation are based on those described by Gamborg et al. (1968) for B5 medium with 20 g/l sucrose and 8 g/l agar, with the pH adjusted to 5.5 with NaOH, and by Murashige and Skoog (1962) with 30 g/l sucrose and 8 g/l agar, with the pH adjusted to 5.7. B5M medium (Swanson and Tomes 1980a) was also used. Callus was initiated from 5-mm hypocotyl sections and plated on B5M callus induction media (B5M + 0.1 mg/l 2.4-D). Cultures were incubated in a growth room held at 25 °C with a 16-h photoperiod and a light intensity of approximately 29 $\mu mol^{m^{-2}s^{-1}}$ of cool white fluorescent light. Subcultures were carried out at monthly intervals.

Selection for resistance

Selection 1: Callus culture. For the selection media, the herbicide Harmony was obtained from E.I. Du Pont de Nemours as an 80% wettable powder (Du Pont 1984). A stock solution was freshly prepared before each experiment and filter-sterilized using a disposable Nalgene 0.20 μm filter. Aliquots of herbicide stock solution were incorporated into autoclaved B5M medium just prior to when the medium had cooled. Pretests were performed to find the appropriate levels of herbicide for selection. A dose-response experiment testing growth inhibition was set up with seven concentrations of the herbicide. Ten pieces of callus, weighing a total of 0.5 g per petri plate, were tested on selection media (25 ml/petri plate) containing 10^{-8} to 10^{-3} *M* Harmony at ten-fold increments plus control. Cultures were incubated in a growth chamber and weighed after 2 weeks. A dosage range was chosen that was just above complete growth inhibition but not lethal.

Resistant cell lines were selected using the same procedure. Ten pieces of callus per plate were transferred to selection media supplemented with increasing increments of the herbicide. The concentrations used were 10^{-7}, 2.5×10^{-7}, 5×10^{-7}, 7.5×10^{-7}, 10^{-6}, and 2.5×10^{-6} *M* Harmony. There were six passages each lasting 14 days. Based on visual observations, only the most viable calli parts (i.e., green and friable) were transferred to the next (higher) selection level. Cytotoxic effects were measured after each passage by taking the fresh weight of the callus.

Selection II: Using shoots regenerated from selection I. After 12 weeks of *in vitro* herbicide exposure, surviving callus sections from the last two subcultures of selection I (10^{-6} and 2.5×10^{-6} *M*) were transferred to regeneration medium (B5M medium supplemented with 0.5 mg/l BA; MacLean and Grant 1987) and incubated under the same conditions as above. Subcultures were performed at 4-week intervals until the regenerated shoots were approximately 2 cm in length. Due to the high number of shoots regenerated, a second selection procedure was performed. Shoots from both treatments (Harmony, control), 1–2 cm in length, were aseptically arranged in alternate rows in two 20 × 35 cm pyrex baking dishes, each containing 750 ml agar selection medium (10^{-6} *M* Harmony) or control medium (Fig. 1 A). The shoots in the pyrex dishes were incubated for 14 days.

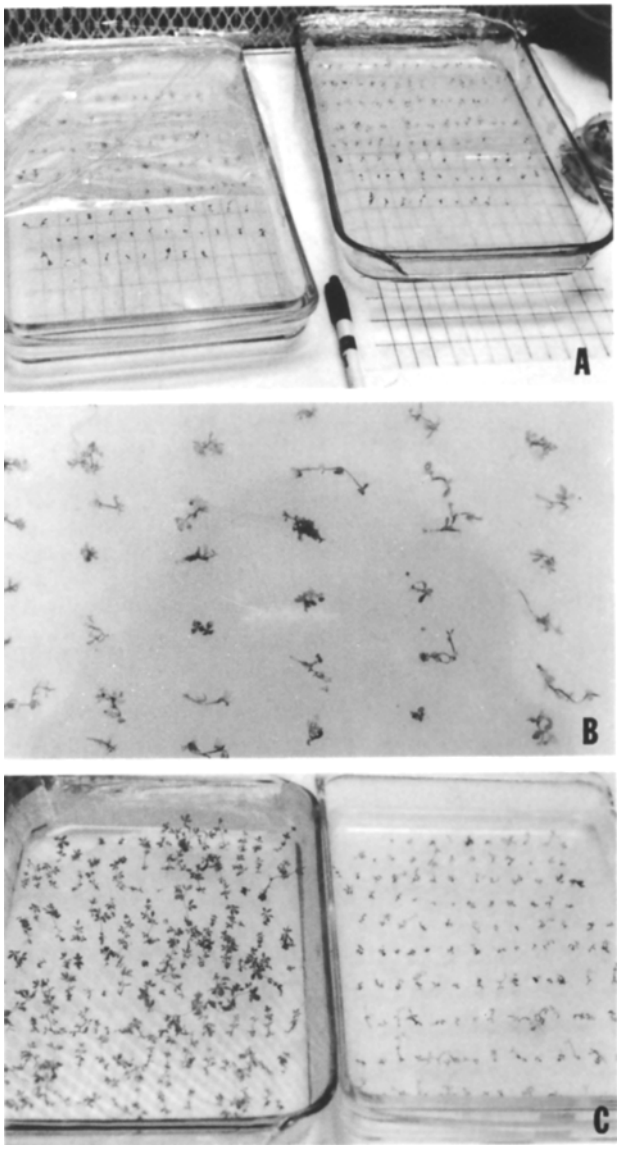

Fig. 1. **A** Regenerated shoots from selection I being placed on control (*left*) and selection (*right*) media. **B** Close-up of shoots on selection media after 1 week of growth. **C** Regenerated shoots placed on control (*left*) and selection media (*right*) after 2 weeks of growth

Surviving shoots were rescued from the *in vitro* herbicide screening procedure and transferred to culture jars containing solidified B5M media with 0.2 mg/l NAA for root induction. Whole plantlets were acclimatized and transplanted to pots with Pro-mix and placed in a growth chamber set for ambient day and night temperatures of 22° and 18 °C, respectively, with a 16-h photoperiod. At the same time, Leo seeds were planted so that seedlings would achieve the same level of maturity as the regenerants to act as controls.

Selection III: Using plants surviving selection II. At the three-leaf stage the seedling controls (A), control regenerants (non-herbicide) from *in vitro* selection (B), and putative herbicide-resistant regenerants from selection media (C) were foliar-sprayed in a Research Instrument Manufacturing Company Spray Chamber

Table 1. Visual rating scale (1 to 10) for phytotoxic symptoms

1. Plant is dead
2. Recovery not possible
3. Stunted and complete apical death
4. Severe necrosis of meristems, recovery doubtful
5. Yellowing and beginning of apical necrosis
6. Yellowing but recovery possible
7. Apical meristems light green and plant stunted
8. Green and less stunted than 7
9. Green and slightly stunted
10. No damage compared to respective untreated controls

(Guelph, Ontario) for further selection. Application to foliage was with a teejet full-cone nozzle (TG 0.7) calibrated to deliver 500 l a.i./ha solution of the appropriate concentration of the herbicide. Plants were visually rated 1 (dead) to 10 (no damage) for phytotoxicity at weekly intervals after treatment (Table 1). Spraying was repeated several times with higher concentrations in the range prescribed in the Du Pont Technical Information Bulletin (Du Pont 1984) to cull the plants and select those with increased tolerance levels. At each selection stage the plants were coded so that their origin and selection route could easily be determined.

Evaluation of resistance

Callus test of putative mutant selections. Callus was initiated from the 12 selected putative resistant regenerated lines, using 5–10 mm shoot tip stem sections, and plated on callus induction medium. For most selections growth was slow (compared with Leo control), whereas for other lines callus establishment was not seen even after several weeks. Therefore, after 6 weeks the explants were transferred to MS medium supplemented with 0.4 mg/l 2,4-D. Cytotoxicity was tested on those lines that produced enough callus. Six pieces of each callus line weighing a total of 200 mg were plated on selection medium with 10^{-5} *M* Harmony, incubated for 2 weeks, weighed, replaced on fresh selection medium, and reweighed again after 2 weeks. A complete randomized design consisting of three petri dishes per cell line per treatment was used for fresh weight measurements at 2- and 4-week intervals and analyzed using standard analysis of variance procedures.

Growth chamber analysis. To test and characterize the level of resistance of putative mutant selections, four cuttings were made of all 12 surviving lines. Control plants grown from seed were treated similarly. At the three-leaf stage, when the cuttings were approximately 6 cm, the plants were foliar-sprayed with herbicide (46.83 g a.i./ha). The plants were arranged in a complete randomized design consisting of three separate pots per plant line, scored visually for phytotoxicity, and the height was measured at weekly intervals for 6 weeks. Data for week 0, week 6, and the height increase over this period were analyzed using standard analysis of variance procedures, followed by multiple comparisons using Duncan's Multiple Range test.

Field analysis I (1989). Eleven putative resistant cell line selections, plus Leo seedlings as controls, were field-tested. Cuttings, 25 days old and approximately 10 cm high, were transplanted into a 12×30 m field plot. Plants were spaced 1 m apart. Ten days after transplanting, each plant was sprayed with herbicide or water (0, 43.86, and 86.54 g a.i./ha) using a hand-operated plant mister that had been previously calibrated. Data were collected for:

(a) Phytotoxicity, at 4-day intervals for 3 weeks by visually ranking injury (Table 1).
(b) Days to flowering and pod maturity, when at least two open flowers per plant and two fully expanded green pods had developed.
(c) Dry weight yield and height after 3 months. Plants were cut at the crown and dried in a corn dryer at 24 °C for 4 days and weighed. Height was measured in the field from the crown to the tip of the average shoot.
(d) Pollen stainability. Flowers (three per plant) just prior to anthesis were collected from all 12 Harmony selections from the field and scored for pollen stainability.

One hundred pollen grains per flower, mounted temporarily in fast green and lactophenol, were scored at a magnification of $400\times$. Pollen grains that stained green and appeared plump were considered viable, whereas unstained and shrivelled grains were considered nonviable (Grant et al. 1962).

Analysis of variance was performed on data collected for yield, height, days to flowering, and days to pod development. Where significant interaction effects between cell lines and herbicide treatments were seen, orthogonal contrast analyses were performed.

Field analysis II. During the summer of 1990, seeds from lines H1–1, H5–1, H5–10 that had been previously germinated *in vitro* and screened on 10^{-5} *M* Harmony selection media were field tested. Leo controls were also tested. The seeds had been collected from the 1989 field experiment from outcrossed lines. The herbicide treatments were 0.0, 30.0, 60.0, and 100.0 g a.i./ha. Analysis of phytotoxicity ranking was performed using the Friedman test for nonparametric data.

Cytogenetic studies. Chromosome numbers were determined for all 12 Harmony lines that had been field tested (1989). Root tips from 15-day-old cuttings were collected and pretreated for 2 h in a 0.002 *M* 8-hydroxyquinoline aqueous solution. The root tips were fixed for 24 h in 95% ethanol and glacial acetic acid (3:1), hydrolyzed in 1 *N* HCl at 60 °C for 10 min, followed by staining in leucobasic fuchsin for 4 h, according to the Feulgen technique (Darlington and La Cour 1976). Stained root tips were treated with 4% pectinase aqueous solution for 4 h to facilitate maceration and stored in 70% ethanol in a refrigerator. Slides were prepared by squashing root tips in a drop of 45% acetic acid. Permanent slides were prepared by the freezing technique of Conger and Fairchild (1953).

Acetolactate synthase enzyme assay. ALS activity was measured using the method described by Chaleff and Mauvais (1984) with modifications by Swanson et al. (1988) and J. Granger (Agriculture Canada, personal communication). The protocol for the assay is as follows.

I. Extraction

1. In a mortar and pestle, 1 g fresh weight of the youngest fully expanded leaves, randomly picked from 10 cuttings of each line, was homogenized in 3 ml extraction buffer containing: 100 m*M* KH_2PO_4 at pH 7.5, 0.5 m*M* $MgCl_2.6H_2O$, 1.0 m*M* Na pyruvate, 0.5 m*M* thiamine pyrophosphate (TPP), 10 μ*M* flavine-adenine dinucleotide (FAD), with the addition of 250 mg polyclar.
2. The solution was centrifuged in a Beckman microfuge for 20 min at 12,500 rpm.

II. Purification

1. Acetolactate synthase was precipitated from the supernatant with an equal volume of saturated ammonium sulfate and left to stand for 1 h.
2. The mixture was centrifuged for 5 min in a microfuge.

3. The pellet was resuspended in extraction buffer base (the first three ingredients of the extraction buffer) and desalted by pipetting the mixture into a Sephadex G-25 column (Pharmacia PD-10) equilibrated with 20 ml of the same buffer. All the above operations were carried out at 4 °C.

III. Enzyme assay

1. In a 1.5-ml Eppendorf tube, 400 µl reaction buffer was added consisting of: 65 m*M* KH_2PO_4 at pH 7.5, 40 m*M* Na pyruvate, 10 m*M* $MgCl_2$, 0.25 m*M* TPP, 23 µ*M* FAD. To this 100 µl column eluent was added plus 5.0 µl of the appropriate herbicide concentration. Assays were carried out in a final volume of 500 µl and incubated for 90 min at 30 °C.
2. The reaction was stopped by adding 200 µl of 6 *N* H_2SO_4 to each tube, and incubated for 10 min at 55 °C to achieve complete conversion of acetolactate to acetoin.
3. To the acidified reaction mixture was added: 150–200 µl 50% NaOH, 150 µl 0.5% creatine, 150 µl 5.0% alpha naphthol (freshly prepared in 2.5 *N* NaOH). The mixture was incubated at 55 °C for 10 min to accelerate color development, then centrifuged in a microfuge at 12,500 rpm for 5 min.
4. Acetoin was measured by reading the absorbance at 525 nm. Final concentrations of 0, 1, 10, 100, 1,000 µg/l of Harmony were used. Protein concentrations were measured using the Bio-Rad Protein Assay reagent with bovine serum albumin as a standard. All measurements were the average of four independent determinations at each herbicide concentration and were expressed as the percent of uninhibited ALS activity (without Harmony). The I_{50} was defined as the herbicide concentration needed to inhibit ALS activity by 50% as compared to respective controls.

In vitro seed germination trials. A successful assay was developed to screen tolerance levels of seeds collected from the field trials. Surface-sterilized, presoaked seeds were plated *in vitro* on selection medium (with 10^{-5} *M* Harmony). Leo control seed readily germinated and their cotyledons opened normally; however, true leaves (trifoliate) and normal root growth developed only on the resistant selections.

A trial using seeds from the seven fertile lines, collected from the outcrossed field (1989) plants, was set up to screen for Harmony resistance using the above procedure. Twenty sterilized seeds per petri plate containing 10 ml of selection medium were germinated in the dark at room temperature for 3 days and then placed in a growth room to develop. Leo seeds, also from the field test, were used as the standard for comparison. This test was performed three times and a total of approximately 100 seeds/line were scored for trifoliate leaf development 2–3 weeks after germination.

Results and discussion

Selection for resistance

Selection I. The selection procedure was discontinued at passage 6 (2.5×10^{-6} *M* Harmony), as a marked decrease in cell division occurred when the herbicide treatment became lethal to a majority of the cultured cells. The callus at this stage was almost entirely brown with some small greenish calli surviving the treatment. These calli were excised and placed on regeneration medium. Shoot regeneration occurred after 1–2 months at approximately the same rate (65–80%) for control and selected lines.

Selection II. The number of shoots regenerated from excised callus areas was very high. Therefore, approximately 400 shoots, 3 cm high, were screened again on selection media (Fig. 1). This selection procedure, which was devised in our laboratory, makes *in vitro* selection more time and space efficient. As a result, one can select for the trait at the shoot stage before root induction and before transplanting to the greenhouse.

Shoots regenerated from callus selection media, supplemented with 2.5×10^{-6} *M* Harmony (passage 6) from selection I, had the highest survival rate in selection II (38.7% versus control 12.1%). Because of the strong selection pressure exerted on the callus tissue in selection I, it was quite unexpected that so many shoots regenerated, and that such a high percentage of the regenerated shoots were not resistant. Since callus is an aggregate of plant cells and could contain both wild-type and resistant cells, regenerating shoots can arise from either cell type in a chimaeric cluster. The wild-type cells would have a competitive advantage since there tends to be selection for normal karyotypes in plant regeneration (Torrey 1967). Alternatively, epigenetic changes may be responsible for the initial resistance response, which is subsequently lost in regeneration or with the removal of the selection agent (Chaleff 1981, 1983).

After this selection step the shoots were placed on solidified rooting medium. Preliminary tests comparing root initiation on media with 0.2 mg/l NAA or media without growth regulator supplements showed no differences in the number of shoots developing roots; however, the roots from each treatment were morphologically very different. Typically, those grown on hormone-free media were long and unbranched with only one root per shoot, whereas roots developing on NAA media were much shorter and thicker with many root hairs, some secondary root branching, and very often more than one root per shoot. As a consequence, the plantlets from the latter group were bushier and more robust with approximately 80% surviving acclimatization and transplantation.

Selection III. As expected, regenerated whole plants that survived *in vitro* selection I and II on selection media (C) had the highest survival rate (36%) after Harmony foliar spray treatment. These results demonstrate that the resistant trait selected for at the cellular level was also expressed by the whole plant. (All of the surviving variants originated from callus from 2.5×10^{-6} *M* Harmony selection medium, passage 6.) Leo wild type from seed (A) and regenerated control plants from *in vitro* selections I and II (B) had similar survival rates; 13.3 and 14.3%, respectively. These results may indicate that the inherent resistance found in the Leo population is constant and stable through tissue culture and regeneration procedures, and that the selection pressure exerted by the her-

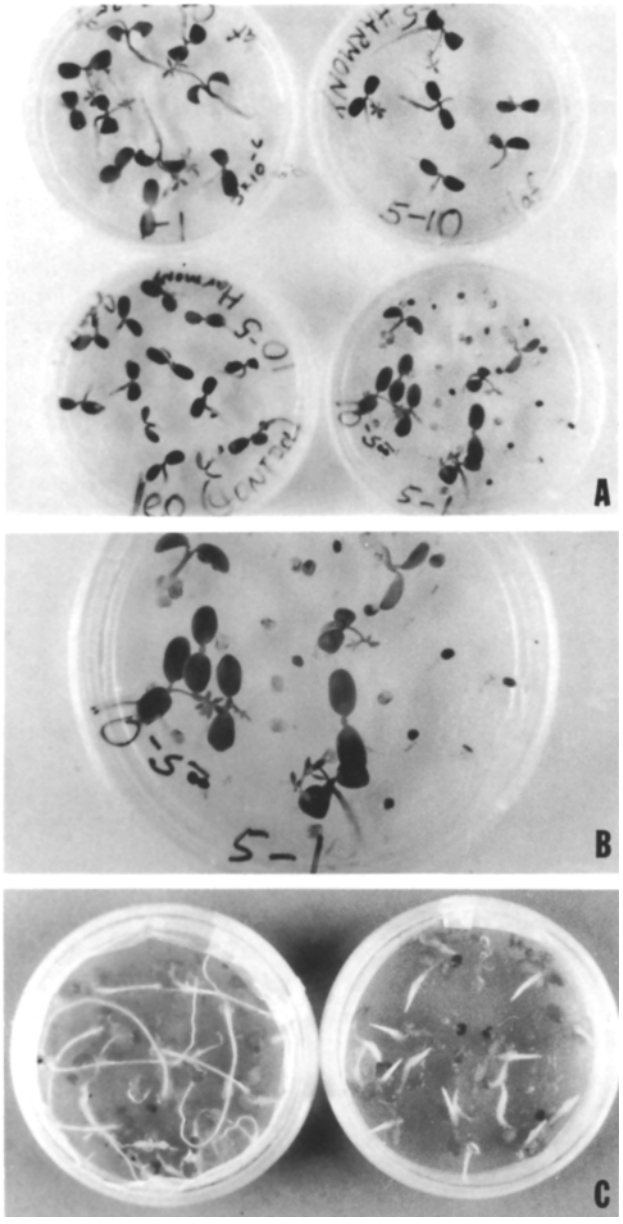

Fig. 2. **A** Clockwise, seeds of Leo and resistant lines 1–1, 5–10, and 5–1 germinating on Harmony 10^{-5} *M* selection media. After 3 weeks of growth, secondary leaf formation is visible on resistant lines but not on Leo (*bottom left*). **B** Close-up of line 5–1. **C** Comparison of root growth of a resistant line (*left*) and Leo (*right*) on Harmony selection media (10^{-5} *M*) after 2 weeks growth

bicide is a necessary selection factor, or is acting as a mutagen and is responsible for the resistance developed in the variant selections. This supports the results of Brettel et al. (1980) and Larkin and Scowcroft (1983), where the recovery of resistant material was greater in the presence of the toxin than when it was dependent only on somaclonal variation (Duncan and Widholm 1986).

Table 2. Effect of Harmony selection medium (10^{-5} *M*) on callus growth of regenerant lines after 2 and 4 weeks. The percent of 0 (control) = fresh weight (g) of treatment (10^{-5} *M*) as a percent of respective control

	2 weeks			4 weeks		
Cell line	0	(10^{-5} *M*)	% Control	0	(10^{-5} *M*)	% Control
(A) Seedling controls						
Leo	0.870	0.619	71.1	1.564	0.647**	41.4
(B) Control regenerants from in vitro selection						
5–14	1.054	0.724	68.6	2.563	0.796**	31.0
(C) Mutant lines from selection media						
1–1	0.784	0.728	93.0	1.656	1.209*	73.0
5–1	0.818	0.769	94.0	1.949	1.703 NS	87.3
5–7	1.138	0.989	86.9	2.246	1.940 NS	86.4
5–10	0.873	0.778	89.0	1.894	1.461*	77.1

**,* Significant at the 0.01 and 0.05 level, respectively, using orthogonal contrast analysis
NS not significant

Evaluation of resistance: at the calli level

Trials of callus from regenerated plants. Harmony, like other sulfonylurea herbicides, functions by inhibiting the acetolactate synthase (ALS) enzyme, which catalyzes the first step in the biosynthesis of the branched-chain amino acids necessary for such primary functions as cell division. Therefore, cell growth of selected lines can demonstrate whether the resistant trait is stable, that is whether or not it is still present at the cell level after regeneration.

Meristematic regions of regenerated plants were recultured to test this hypothesis. After 6 months of callus growth, enough material was accumulated from 6 of the 12 lines to perform a trial. Resistance at the cell level had been retained approximately 18 months after the initial selection and also through regeneration for the lines H1–1, H5–1, H5–7, and H5–10 (Table 2). One line 5–7, which was not included in the field trials because cuttings grew slowly, was very resistant at the cell level.

Evaluation of resistance: at the whole plant level

Growth chamber and field data analyses: Phytotoxicity. Injury caused by postemergent spraying was first expressed in the unselected controls as severely stunted growth, followed by leaf chlorosis and meristem necrosis, whereas in selected lines some stunting and only slight yellowing of the leaf meristems were observed. In addition to these initial symptoms, after approximately 6 weeks in the growth chamber the selected variants (C) recovered, whereas the other selections, controls (A) and

Table 3. Indoor study: effect on height prior to (week 0) and 6 weeks after herbicide application to cuttings of selected regenerant lines

Cell line	Height (mm)* Week 0	Height (mm) Week 6	Increase in height
(A) Seedling controls			
3–6	11.0 ab**	14.0 de	3.0 efg
5–12	9.8 ab	10.7 e	0.9 g
Leo	10.4 ab	11.8 e	1.4 fg
(B) Control regenerants from in vitro selection			
5–7	10.0 ab	16.7 cd	6.7 cde
5–14	8.3 b	9.3 e	1.0 fg
(C) Mutant lines from selection media			
1–1	11.7 a	17.7 cd	6.1 def
4–16	9.5 ab	30.6 a	21.1 a
5–1	9.5 ab	19.8 bc	10.3 bcd
5–10	12.3 a	31.7 a	19.4 a
Leo (untreated)	12.5	27.3	14.8

* Mean height is based on the average of three replications of each cell line at each treatment. Harmony application rate: 43.76 g a.i./ha

** Means followed by the same letter, within columns only, are not significantly different at the 0.05 level according to Duncan's Multiple Range Test

Table 4. Least significant difference (LSD) for multiple comparisons on mean injury rating of simple effects of genotypes at each herbicide level (H1, H2, and H3[a]) from field trial 1990

Cell line	H1		H2		H3	
	Mean rank	Actual rating	Mean rank	Actual rating	Mean rank	Actual rating
(A) Control lines from seed						
Leo	1.0 a	(3.18)	1.0 a	(2.2)	1.0 a	(2.54)
(C) Mutant lines from selection media						
1–1	3.4 b	(7.0)	3.3 b	(7.2)	3.1 b	(6.36)
5–1	2.6 ab	(6.6)	2.8 b	(7.4)	2.7 ab	(5.64)
5–10	3.0 b	(6.52)	2.9 b	(6.36)	3.2 b	(5.68)
Chi-squared	9.96*		9.42*		9.42*	
TSS	16.6		15.7		15.7	

[a] H-1, H-2, H-3 = 30, 60, 100 g a.i./ha Harmony, respectively

* Chi-squared values significant at the 0.05 level

Values in parentheses are the means of five replications of nine combined ratings over a 3-week period that followed herbicide application

LSD for multiple comparisons = 9.14. Mean ranks followed by different letters in the same column are significantly different at the 0.15 experiment-wise error rate

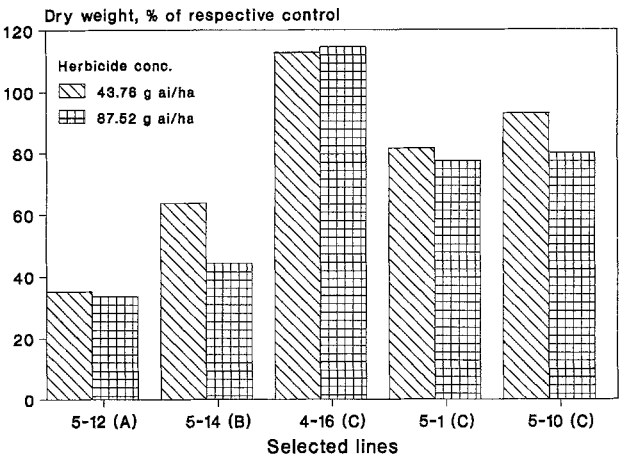

Fig. 3. Dry weight yield as a percentage of respective controls in response to a foliar application of Harmony to *A* control plants grown from seed, *B* control plants grown from tissue culture, and *C* selected resistant lines grown from tissue culture

(B), lost most of their leaves and were extremely stunted (Table 3).

The field data for 1989, however, is less conclusive in that a large percentage of the plants, including Leo controls from seed (A), recovered and grew vigorous new tillers following an initial period (4–5 weeks) of severe phytotoxic damage. Similar results for field-tested, transformed SU tobacco plants have been reported (Knowlton et al. 1988). These workers observed that with time, non-transformed plants began to recover and new growth was initiated. In our study, a significant yield drop in controls occurred as a result of the herbicide application. Harmony selections H2–14, H2–12, H4–16, H5–1, H5–10 had the lowest injury ratings in both growth chamber and field experiments.

Field data collected during the summer of 1990 were more conclusive. Fertile selections H1–1, H5–1, and H5–10 survived herbicide treatments up to 100 g a.i./ha, approximately four times the recommended field rate as compared to Leo controls, which died at 30 and 60 g a.i./ha (Table 4). The different response observed in 1989 and 1990 may have been caused by the very hot and dry period in May 1990. The reaction of wild-type plants to the herbicide, in combination with the drought stress, may have been too traumatic, and consequently most of the control plants died. Grant and Marten (1985) and Madill et al. (1980) noted that herbicides can suppress growth of trefoil and cause serious crop damage under conditions of drought or high temperature. Blair and Martin (1988) discuss the fact that high temperatures enhance the effects of chlorsulfuron and metsulfuron-methyl activity in sensitive species such as *Sinapus alba*, while this is reversed with tolerant species such as wheat. Results from field-grown, *in vitro* selected plants demonstrate that there is a gradient of tolerance in trefoil. These

field trials also emphasize the importance of testing selected mutants under varying agronomic conditions.

Yield. From the analysis of the dry weight yield, which was measured after 3 months growth in the field (1989), it is evident that the herbicide application affected overall forage yield. The dry weight of selected variants (C) was less affected by the herbicide application than wild type from seed (A) or control regenerants from culture (B) (Fig. 3).

Flowering and fertility. As expected, several of the selections were sterile. Five lines – H1–10, H2–14, H4–12, H4–16, and H4–17 – produced flowers but pod and seed formation were aborted. Also, these variants appeared to be less fit than the wild type. Gressel et al. (1978) have attributed this problem to tissue culture techniques and somaclonal variation causing multiple mutations, many of which are deleterious. In general, fertility has been a major problem with cell-culture-dervied, herbicide-resistant plants. However, three lines – H1–1, H5–1, and H5–10 – with increased herbicide tolerance levels were fertile. Morphologically, these plants are very similar to Leo for most quantitative traits (data not shown).

Flowering was significantly delayed by herbicide application to Leo, control plants (A) and control regenerants (B), but not to the putative mutant selections (C). In a similar experiment, Swanson et al. (1988) noted that maturity was delayed in *Brassica napus* regenerants when 6 g/ha chlorsulfuron were incorporated in the soil. Jordan and McHughen (1987) reported comparable results with flax regenerated from chlorsulfuron-resistant selected callus. Delay in flowering to a period of less than optimal conditions can lead to seed yield reductions and insect/seed problems in trefoil (P. R. Beuselinck, personal communication).

In vitro seed germination tests. From Table 5 and Fig. 2 it may be concluded that the herbicide-resistant trait is heritable. Germination of seeds with the resistant genotype was considerably lower than for wild-type seeds. The reasons for this are not immediately evident, but a lower fertility rate, an increased susceptibility to insect or microorganismal damage, or a shortened shelf life are some possibilities.

Since *Lotus corniculatus* is an outcrossing species and the seeds used for this test were from outcrossed plants collected from the field experiment, it is difficult to surmise inheritance patterns without a controlled genetic study. Crosses are now in progress using fertile selections from the field; however, genetic analyses with outbreeding polyploids is exceedingly difficult and the results may still not be conclusive.

Cytogenetics. Chromosome counts of the 12 Harmony selections showed that of the five infertile selections, four are in the octoploid range ($2n = 8\times = 48$) and one (H4–12) was aneuploid with approximately 45 chromosomes. Not surprisingly, all the selections that flowered and produced seed had the normal complement of chromosomes ($2n = 4\times = 24$).

Table 5. Effect of herbicide on in vitro seed germination

Cell line	N tested	N resistant[a]	% resistant
(A) Control lines from seed			
Leo	164	3	1.8
3–6	78	1	0.6
5–12	118	1	0.9
(B) Control lines from (nonherbicide) in vitro selection			
5–14	54	1	1.9
(C) Mutant lines from selection media			
1–1	137	56	40.9
4–10	160	3	1.9
5–1	101	36	35.6
5–10	74	26	35.1

[a] Seeds that germinated and grew secondary leaves on selection media with 10^{-5} *M* Harmony

Other studies using *in vitro* selection of trefoil have also produced regenerants with gross phenotypic and genotypic abnormalities. In this laboratory, MacLean and Grant (1987) found that 20% of callus culture regenerants had aneuploid, mixoploid, or polyploid chromosome numbers. Tomes (1982) reported similar results where 20% of the trefoil regenerants were polyploid, several of the 'normal' regenerants had reduced male fertility and seed set, and a number of the regenerants failed to flower.

Chromosome number changes involve other phenotypic changes such as large leaves, dark green coloration, reduced petiole length, thick internodes, and large florets, characteristics associated with polyploids (Bingham and McCoy 1986). In these selections polyploidy was associated with all of the above morphological changes as well as stunted growth habit and lower dry-weight yield.

Acetolactate synthase enzyme assay. ALS assays were carried out to determine whether a mutant ALS was the basis for the Harmony resistance. ALS inhibition of mutant selections (H1–1, H5–1, H5–10) was significantly lower than Leo controls, according to Duncan's test for comparison of means. It may be that a mutant ALS gene with an altered binding site is responsible. However, this is not certain since most gene mutations associated with increased SU resistance cited in the literature describe ALS resistance levels of 10- to 1,000-fold that of wild-type levels (Chaleff and Mauvais 1984; Haughn and Somerville 1986; Swanson et al. 1988; Saxena and King

1988). A CS-resistant tobacco mutant isolated from cell culture had an I_{50} value that was >8000 n*M* CS compared to 14 n*M* for the wild type (Chaleff and Mauvais 1984). The I_{50} values for ALS inhibition for CS-susceptible and -resistant *Kochia* biotypes were 22 and 400 n*M* CS, respectively (Saari et al. 1990). On the other hand, Sebastian et al. (1989) described the CS soybean mutant W20 as resistant, which had only a threefold greater enzyme activity than the wild type remaining at 200 µg/l CS. I_{50} values were 10 and 24 µg/l CS for sensitive and resistant soybean lines, respectively. In our study, the percentage ALS activity remaining for lines H5–1 and H5–10 at the highest herbicide concentration tested, 1,000 µg/l, was only twice the activity in Leo (Fig. 4). At 100 µg/l, there was approximately a threefold increase in activity remaining for selection H5–10 as compared to Leo. I_{50} values were 2.5 and 5.5 µg/l Harmony for Leo and line H5–10, respectively. A similarity does exist with the Sebastian et al. (1989) study, and it is interesting to note that in both studies selection is for plants of the legume family.

Alternatively, an amplified ALS gene may be responsible for the differential tolerance in selection H5–10, where the protein levels were found to be higher than the wild type. Total protein in enzyme extracts was 4.95 ± 0.30 mg/ml for H5–10 and 4.05 ± 0.39 mg/ml for Leo. Meredith (1984) has suggested that a step-wise selection procedure, as used in this experiment, may favor the selection of genotypes resulting from gene amplification or mutations in organelle genomes. This phenomenon was also seen in bacteria and in cultured animal cells, where specific genes can undergo amplification when the cells are placed under selective conditions (Stark and Wahl 1984).

In conclusion, the resistant phenotype is present in callus derived from regenerated lines, in seedlings and in plants from outcrossed seeds, establishing that resistance is expressed at all stages of development and is heritable. The field trial results demonstrate a high degree of resistance, as selected lines were able to continue to grow and mature without any serious setbacks after herbicide treatment, whereas severe morphological and physiological damage to wild-type plants was seen to accumulate, affecting maturity, pod formation, and final yield. This normal development is important for herbicide-resistant birdsfoot trefoil crops that would be grown for forage and for seed. ALS enzyme assays indicate that at the biochemical level, the resistant lines H1–1, H5–1, and H5–10 are significantly more tolerant to Harmony than Leo wild type. It cannot, however, be concluded that ALS activity is the biochemical mechanism responsible for the resistant phenotype of these trefoil selections until further studies are performed. One has to examine other mechanisms of herbicide resistance such as gene amplification, detoxification systems, or physiological processes

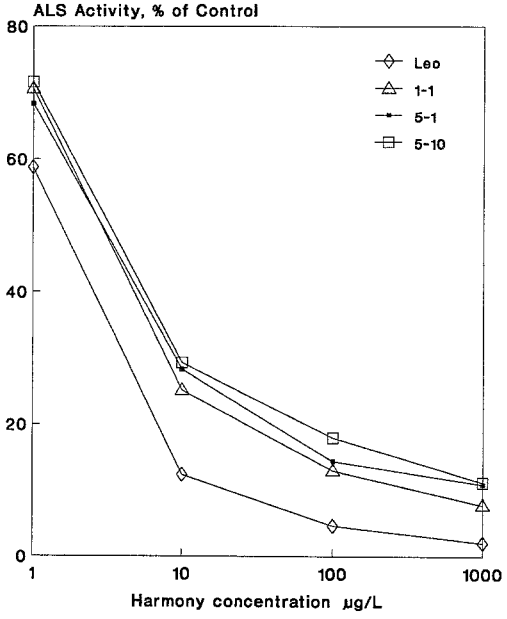

Fig. 4. Percentage of acetolactate synthase activity (as a percent of respective controls) remaining in wild-type (Leo) and mutant lines 1–1, 5–1, and 5–10 leaf extracts at increasing concentrations of Harmony

that can be achieved by the plant. It still remains to be defined in what way these selected variants have achieved herbicide resistance. The different degrees of resistance displayed in this study by the resistant variant cell lines H1–1 and H5–1, H5–10 in callus and seed germination tests suggest that mutations may have arisen independently and that separate mutations may be found for trefoil ALS gene(s) that confer differential herbicide sensitivity. Investigating these plants at the molecular level may answer some of these questions. Furthermore, seeds of selected lines selfed and backcrossed with Leo may elucidate much of the needed information on the inheritance patterns, linkage groups, and type of gene(s) involved.

Despite the drop in overall yield of the tissue culture selections, these lines can be useful in heavily weed-infested fields, where the increased safety margin between crop and weed sensitivity is important. Alternatively, their use in conventional breeding programs with trefoil or other *Lotus* species for improved SU resistance or as an identifiable marker in future scientific research with birdsfoot trefoil is important. Also, on the plus side is the fact that Harmony is a herbicide that is highly effective, safe, and rapidly biodegraded with low soil residues, and therefore, obtaining a tolerant crop species would minimize potential damage to the environment.

On the other hand, weed infestation is one of the problems that might occur from these regenerants, since tolerance of the wild type to the SU herbicides at low levels is somewhat inherent (ALS assay, field test 1989).

Furthermore, since birdsfoot trefoil is an outcrossing species, possible exchange with wild relatives can also be detrimental from an evolutionary perspective, where the development of weeds from the crop itself is not unlikely.

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