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The cost of herbicide resistance in white-chicory: ecological implications for its commercial release

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Abstract Applications for the commercial release of herbicide-resistant crops, most of them transgenic, are likely to become more frequent in the coming years. The ecological concerns raised by their large scale use call for risk-assessment studies. One of the major issues in such studies is the relative fitness of the resistant line compared to the susceptible when no herbicide is applied since this will largely determine the long-term fate of the resistance gene outside of the field. Here we report on a comparison of a sulfonylurea-resistant line of white-chicory regenerated from a non-mutagenized cell culture with a supposedly isogenic susceptible biotype. The plants were grown in experimental plots at a range of densities in a replacement series. The reproductive output of the plants decreased with increasing density but no significant difference was found between the two lines for any vegetative or reproductive trait at any density. This suggests that no cost is associated with the mutation causing the resistance and that the resistance gene would not be selected against if it escaped to populations of wild chicories.

Key words Herbicide resistance · Sulfonylurea · Fitness · Risk assessment · Chicory

Introduction

Two transgenic herbicide-resistant crops have been recently authorised for commercialisation in the European Union, a Bromoxynil-resistant line of tobacco and a Basta-resistant line of rapeseed. Applications for the commercial use of herbicide-resistant crops, whether transgenic or not, are likely to become more frequent in the next 5 years and, independently of the transformation technique, the widespread use of these crops raises some environmental concerns. The two major concerns are (1) the possibility that the crop itself will become a nuisance, and (2) the possible development of new resistant weeds resulting from gene flow between the resistant crop and some wild related species. Several factors will govern the evolution of the resistance gene in wild populations.

In the short term, the factors most likely to have an effect are those controlling gene flow between field and wild populations, i.e. seed and pollen migration, selfing rates, and hybridisation barriers between the crop and its wild relatives. These factors are affected by both biological and agricultural practices. It is, however, the relative fitness of the resistant biotype compared to the susceptible in the absence of herbicide that has been recognised as the prime factor determining the long-term fate of the resistance gene. If the relative fitness of the resistant biotype is lower than that of the susceptible, its frequency should decrease with time if no herbicide is applied. This relative decrease of fitness is named the cost of resistance (Warwick and Black 1994) and is expected to result from modifications of the physiological pathways which confer the resistance. It has been investigated for a number of species and is thoroughly reviewed in Warwick and Black (1994). This study demonstrates that the existence of such a cost is highly dependent on the plant species, the type of resistance, the trait measured, and the environmental conditions.

Sulfonylurea herbicides act by inhibiting the acetolactate synthase (ALS), the first enzyme specific to the branched amino-acid pathway (Chaleff and Mauvais 1984). In weeds, the first reported resistance to a sulfonylurea is dated 1986 (Darmency and Gasquez 1990) and resistant biotypes of *Kochia scoparia* and *Lactuca serriola* have since been reported to cause weeding problems after 3–5 years of herbicide treatment and to spread fast. Sul-

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fonylurea resistance is now avaliable in a few crop species: tobacco (Chaleff and Ray 1984; Brandle and Miki 1993), flax (Jordan and McHugen 1987), sugarbeet (Saunders et al. 1992), maize (Anderson and Georgeson 1989) rapeseed (Swanson et al. 1988; Miki et al. 1990) and carrots (Caretto et al. 1994). When selected by herbicide pressure, whether in weeds or crops, this resistance is almost always due to the production of a mutant ALS less sensitive to the herbicide; this mutation confers very high levels of resistance (Shaner 1991; Saunders et al. 1992; Lavigne et al. 1994). Resistance due to gene amplification is however also possible and was reported by Caretto et al. (1994) on *Daucus carota* and Harms et al. (1992) on tobacco.

Studies comparing fitness-related traits of resistant and susceptible biotypes are reviewed in Warwick and Black (1994) and it can be concluded that no general pattern can be drawn about Sulfonylurea resistance. On *K. scoparia*, for example, Mallory-Smith et al. (1992) and Thompson and Thill (1992, in Warwick and Black 1994) found no evidence for a decreased competitive ability in resistant biotypes originating from Kansas and North Dakota, the resistant biotype from North Dakota even tended to accumulate more biomass than the susceptible one. In the same species, Dyer et al. (1993) report that a resistant biotype from Montana germinated faster than the susceptible one in the absence of herbicide. In contrast, Christoffoleti and Westra (1991, in Warwick and Black 1994) report that a resistant biotype from Colorado appeared less competitive than its susceptible counterpart, both in the greenhouse and in field experiments. The main other fitness study concerned with this type of resistance in a weedy species is by Alcocer-Ruthling et al. (1992a,b) who employed a resistant biotype of *Lactuca serriola* from Idaho. The susceptible plants produced more above-ground biomass in competitive growth studies but the two biotypes were similar with regard to seed fecundity, viability and longevity.

One difficulty with such fitness studies lies in the lack of knowledge on the genetic background of the resistant and susceptible biotypes which makes it difficult to separate the effect of the gene itself from that of other genetic differences between the two biotypes. Some studies on resistance to triazines, another family of herbicides, have been conducted using nearly isonuclear lines to control for genetic background effects. The results supported previous findings showing a reduced fitness of the resistant biotypes in species as distinct as *Brassica napus* (Gressel and Ben Sinai 1985; Beversdorf et al. 1988), *Senecio vulgaris* (Mc Closkey and Holt 1990) and *Setaria italica* (Reboud and Till-Bottraud 1991). Reboud and Till-Bottraud, however, note that since the resistance is encoded in the chloroplast and the nuclear background originates from only one parent, the consequences of nucleo-cytoplasmic interactions cannot be assessed in such experiments. Such interactions have been shown to have an effect on plant fitness (Stowe and Holt 1988). Resistance to sulfonylureas differs from resistance to triazines in that it is carried by the nuclear genome. Therefore studies comparing lines with otherwise common nuclear and cytoplasmic genomes can be conducted. McHugen and Holm (1991) report on such a study performed on a transgenic sulfonylurea-resistant flax (*Linum usitatissum*). The two biotypes have similar agronomic performances.

At a more practical level, conflicting interests might arise regarding the cost of resistance when considering herbicide-resistant crops. For farmers and seed producers, it is necessary to grow crops that exhibit high vegetative and seed outputs. However, in terms of risk assessment, it would be safer to consider resistant crops that are as productive as susceptible ones under field conditions (i.e. low competition) but that are depressed outside the field, in particular, under higher competition.

Considering this, in this paper we report on a comparison between some fitness-related traits of a sulfonylurea-resistant biotype of white-chicory (*Cichorium intybus* cv Witloof) obtained by in vitro selection (Lavigne et al. 1994) with those of an otherwise supposedly isogenic susceptible biotype at a range of growing densities. This was done in order to determine the effect of competition on the expression of the possible cost of resistance.

Material and methods

Chicory lines

The resistant line results from in vitro selection on cell cultures. The selection agent was chlorsulfuron, an active ingredient from a sulfonylurea herbicide. The level of resistance is very high and its genetic determinism is monogenic and partially dominant. The resistant individuals exhibit cross-resistances to at least seven other sulfonylureas and one imidazolinone so that the resistance is probably due to a mutant acetolactate synthase (the target enzyme) (Lavigne et al. 1994). The resistant individuals we tested were homozygotes.

The competition experiment

The competition experiment was performed in an experimental field. The plants were grown in stands of 1 m×0.5 m separated by margins of 1 m. No nutrients were added during the experiment. Five different densities were used: 2, 12, 24, 48 and 64 plants/m^2. The replacement series consisted of three planting ratios at each density: 100:0, 50:50 and 0:100. One replicate for each combination of density ratio was set in each of the three blocks. The densities were first determined randomly in one block and then repeated in the two other blocks. The planting ratios were then set at random in each combination of block×density. In the mixed stands, the resistant and the susceptible plants were alternated in each row. As white-chicory is a biennial, measurements on the vegetative growth of the plants were made on the first year and reproductive output was considered in the second year. To avoid frost damage, the plants were kept in the dark in a cold room (4°C) during the winter. They were transplanted back to their original position at the beginning of spring. This provoked the death of about 10% of the plants, evenly distributed among densities and treatments.

Every plant was taken into account during the first year. In the second year, the number of plants studied per stand was reduced to four at the 12 pl/m^2 density, six at the 24 pl./m^2 density and ten at the two highest densities; only plants from the centre of the plots were considered in the three highest densities to avoid possible edge effects.

Estimates of the vegetative biomass were the number of leaves and the height of the plants. They were assessed twice. At harvest a

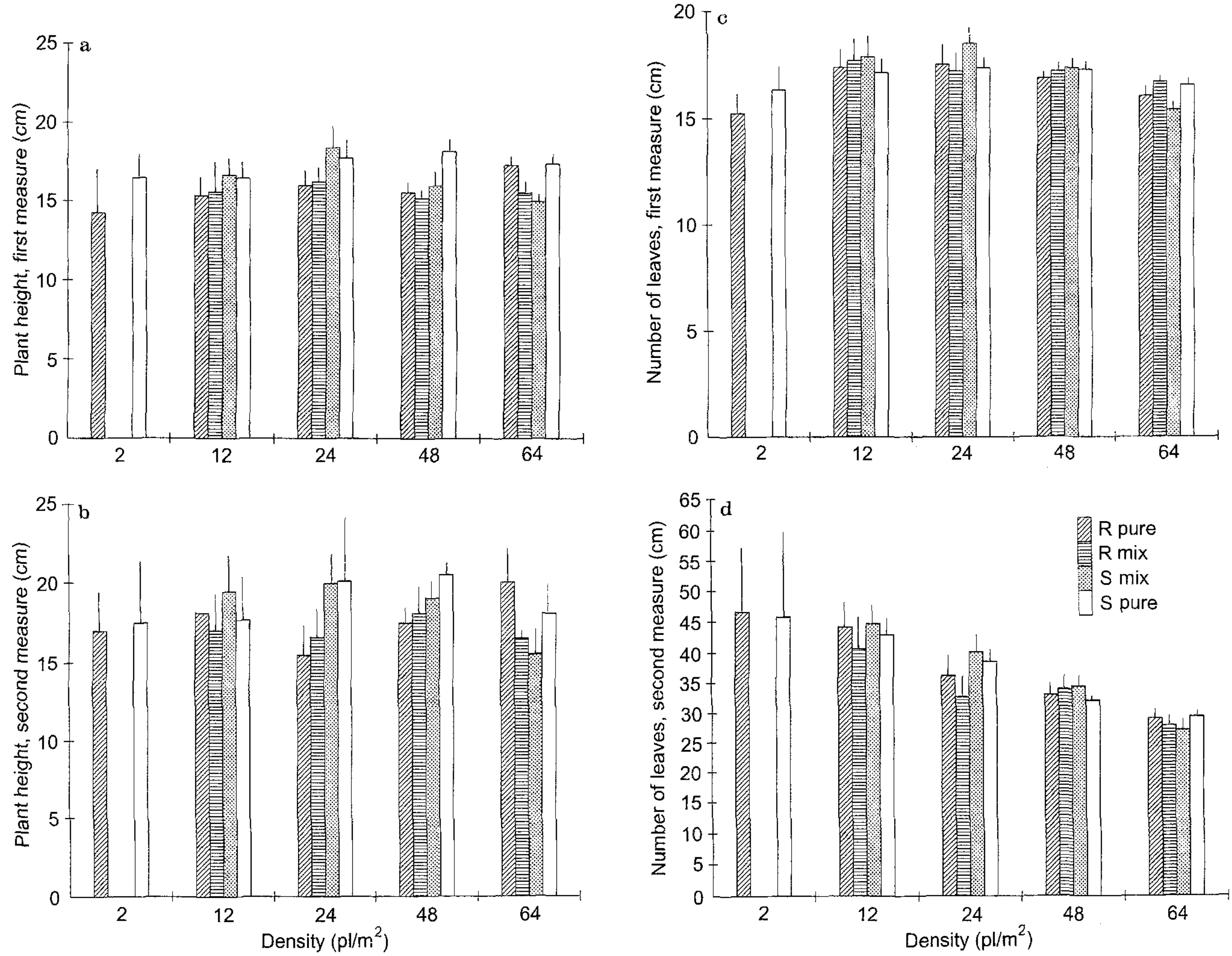

Fig. 1a–d Mean and 95% confidence interval of the vegetative trait measured as a function of density (*R*=resistant biotype, *S*=susceptible biotype, *pure*=stand grown with one biotype, *mix*=stand grown with both biotypes). **a** Plant height (first measure); **b** plant height (second measure); **c** number of leaves per plant (first measure); **d** number of leaves per plant (second measure)

comparison of seed production was inferred from the dry biomass of the glomerules and the total length of the inflorescence. The total length of the inflorescence has previously been shown to be highly correlated with seed production in these lines (Lavigne 1991).

Pollen production

Pollen counts were performed on nine individuals of each biotype (81 and 84 flowers for the susceptible and the resistant biotypes respectively).Three to five flowers were considered per capitulum.

Extraction. Pollen extraction was performed using the method of Loublier et al. (1986) adapted to chicory. It consists of a sulphuric-acid digestion of young anthers followed by a centrifugation that separates the pollen from the anther fragments. For each flower, a volume of 200 μl was added to the pellet. One microliter of this solution was deposited on a Malassez slide and the pollen it contained was counted. Six repeats were made per flower.

Statistical analysis. An analysis of variance was performed on log-transformed data with a hierarchical model: Y=RS+plant(RS)+capitulum(RS×plant)+flower(RS×plant×capitulum) where RS is a fixed effect (resistance or susceptibility) and 'plant', 'capitulum' and 'flower' are random effects.

Pollen efficiency

Pollen efficiency was indirectly assessed by fertilising susceptible plants with a similar amount of pollen originating from homozygous resistant and susceptible plants and thereafter screening the progenies for resistance. All plants were grown at a similar density in an experimental field.

Castration. To prevent selfing, capitula from the recipient susceptible plants were first castrated by rubbing the mature stamens with a wet paint brush. This has been shown (Cordonnier 1990) to kill the pollen grains. The capitula were then bagged and left to dry for about 15 min.

Crosses. Thirteen capitula were not pollinated to control for the occurrence of selfing. For each cross, four flowers from the periphery of a capitulum were first taken from a susceptible and a resistant individual. They were then gathered and rubbed on the castrated capitula. The bags were replaced immediately afterwards. A total of 25 crosses were made. The seeds were harvested at maturity.

Proportion of resistant progeny. Five months after the harvest, seeds were set to germinate in sand pots in a green house. Nutrients were

Table 1 Analysis of variance comparing the pollen production of the resistant and the susceptible biotypes. RS=biotype (resistant or susceptible), PL=plant, CAP=capitulum and FL=flower

Item	Source:		Denominator		F-Value	Pr > F
	DF	Mean square	DF	Mean Square		
RS	1	1283.87	19.37	931.71	1.378	0.2547
PL(RS)	15	1003.11	53.16	851.71	1.178	0.3171
CAP(RS×PL)	49	906.69	98.05	422.76	2.145	0.0007
FL(RS×PL×CAP)	99	415.40	793.00	90.11	4.610	0.0001

supplied once a week by replacing the irrigation water by a nutritive solution. In each pot, ten susceptible and ten resistant homozygous seeds were grown as a control. Six weeks after the germination, the irrigation water was replaced by a chlorsulfuron solution simulating a 10 g a.i./ha treatment. This is five-fold the dose that kills susceptible individuals (Lavigne et al. 1994) but leaves resistant individuals intact. Death rates were assessed 8 weeks later.

Seed germination

One-thousand and two-hundred seeds of each biotype (four replicates of 50 seeds each from six plants) were set to germinate on a filter paper imbibed with water in Petri dishes. The temperature was maintained constant at 18°C and the photoperiod was 8 h dark:16 h light. Seeds were considered as germinated when the radicle was about 2 mm long. The proportion of germinated seeds were arcsine transformed before performing a Student test to compare the resistant and the susceptible biotypes. For each biotype, the speed of germination was also estimated as the mean number of hours necessary for a seed to be noted as having germinated. The comparison was performed with a t-test after log-transformation of the data.

Results

Vegetative biomass

Figure 1 presents the means and 95% confidence intervals for every measurement as a function of density. Density did not significantly affect either the height of the plants or the number of leaves when first measured. On the other hand, the total number of leaves at the end of the first growing season significantly decreased with increasing density. Susceptible and resistant individuals were most often non-significantly different. Out of the six differences that were significant, five were in favour of the susceptible line. A sign test was performed since the number of comparisons was too low for a chi-square test. It indicates that this result is consistent with the fact that the susceptible and the resistant are similar. On a whole, out of a total of 36 comparisons, 26 were in favour of susceptible individuals which is not significantly different from the 18:18 that is expected if susceptible and resistant individuals are similar ($\chi_2=2.46$, $P>0.05$). The same pattern was obtained when the plants were grown in a mixture or in pure stands suggesting that the two biotypes were equally competitive.

Pollen production

The analysis of variance (Table 1) performed on the number of pollen grains counted per μl indicated significant differences between flowers within each capitulum and between capitula within each plant for this trait. There was, on the other hand, no significant difference in the amount of pollen counted either between plants within a biotype or between the resistant and the susceptible biotypes.

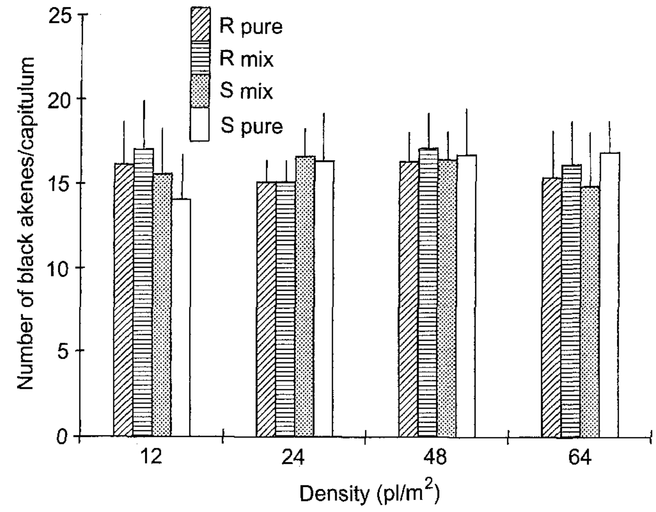

Fig. 2 Mean and 95% confidence interval of the number of black akenes per capitulum at harvest as a function of density (*R*=resistant biotype, *S*=susceptible biotype, *pure*=stand grown with one biotype, *mix*=stand grown with both biotypes)

Pollen efficiency. The 13 capitula that were not pollinated gave a total of six viable seeds. Since there is a competitive advantage to allopollen in this species (Coppens d'Eeckenbrugge et al. 1987), it could be concluded that our method of castration was efficient and that little selfing occurred in the 25 crosses that were performed. Twenty of these gave viable seeds. Among their progeny (86 plantlets), the ratio of resistant versus susceptible individuals was 48:38. A chi-square test indicated that this was not significantly different from the 43:43 expected.

Seed production

From the dry weight of the glomerules

The first step was to check that the number of seeds per glomerule was similar whatever the treatment and the plant type. We could then compare the dry weights of the glomerule and draw conclusions about seed production.

Table 2 Values of the t(obs) in the mean comparisons between resistant and susceptible individuals. A t(obs) superior to the t(theor) means that the two biotypes are significantly different (*⇔1%<P<5%).

Density	t(theor)	Dry weight of glomerules		Total inflorescence length	
		pure stand	mixed stand	pure stand	mixed stand
12 pl./m2	2.074	1.116	0.811	2.053	1.287
24 pl./m2	2.03	0.443	1.204	0.566	2.478 * (S>R)
48 pl./m2	2.01	1.746	0.807	1.485	2.091 * (S>R)
64 pl./m2	2.01	2.251 * (R>S)	0.109	1.043	0.462

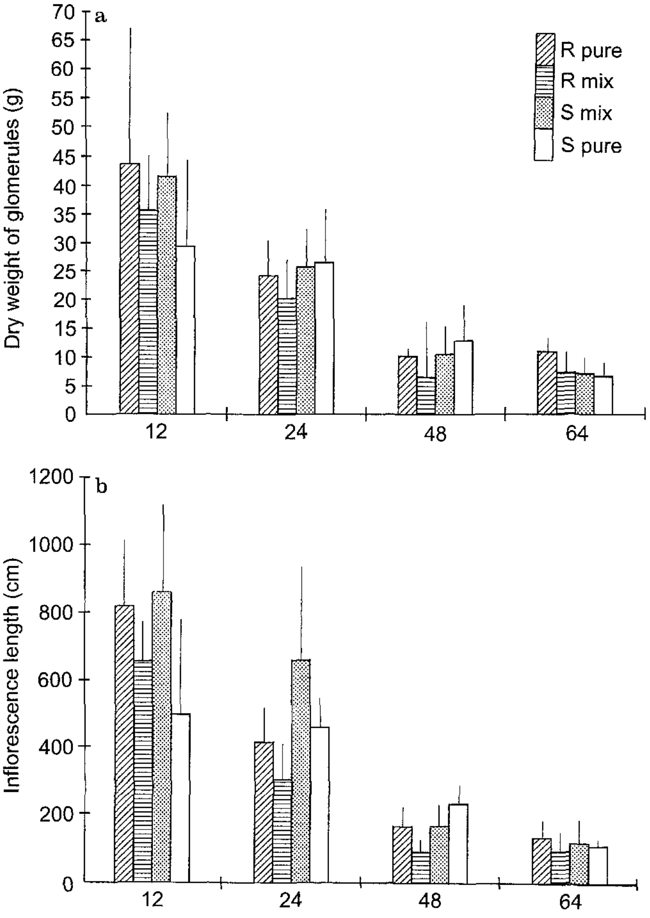

Fig. 3a, b Mean and 95% confidence interval of the two traits used to estimate seed production at harvest as a function of density (*R*=resistant biotype, *S*=susceptible biotype, *pure*=stand grown with one biotype, *mix*=stand grown with both biotypes). **a** Dry weight of the glomerules, **b** inflorescence length

Seeds per capitulum. Two kinds of akenes are produced by chicories. Only the black ones carry a viable seed. The number of black akenes per capitulum are presented in Fig. 2. There were no significant differences between resistant and susceptible lines whatever the treatment. The mean number of black akenes was therefore averaged over every treatment (16±3.55, n=180). The mean number of flowers per capitulum being 25.04±1.54, n=70, the proportion of flowers giving viable seeds was around 74% since each flower can produce at most one seed.

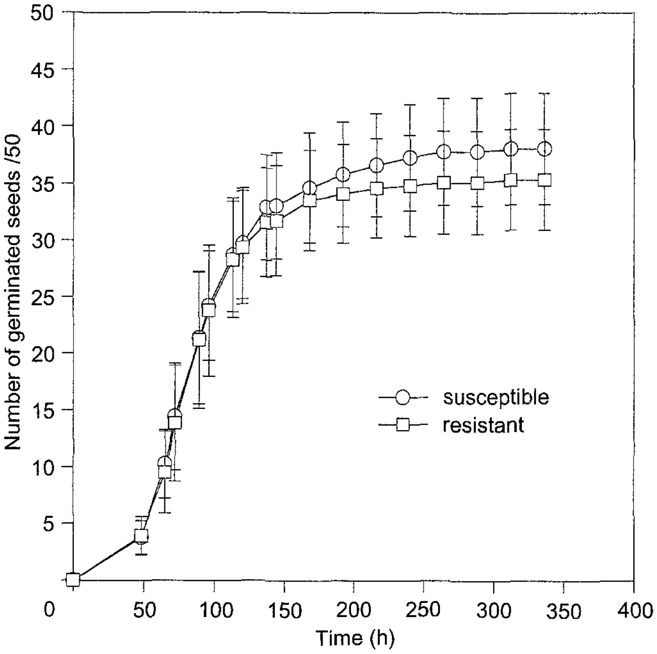

Fig. 4 Mean and standard error of the number of germinated seeds (out of 50) as a function of time for the resistant and the susceptible lines

Capitula per glomerule. On the main axis, the number of capitula per glomerule was similar for resistant and susceptible individuals (2.85±1.30 n=60 and 2.90±0.83 n=45 for resistant and susceptible biotypes respectively, P>0.05). The number of capitula per glomerules was not significantly different whether on the main axis (2.86±0.82 n=70) or on the lateral ramets (3.04±0.71 n=70). The mean was therefore averaged at 2.95(±0.77, n=140).

Glomerule dry weight per plant. These two preceding results indicated that the dry weights of the glomerules per plant was a good estimate of seed production. The results are presented in Figure 3a. Increasing density significantly decreased the mean glomerule dry weight. At the highest density, the resistant biotype had a higher dry weight of glomerules than the susceptible one in pure stands (Table 2). Out of the eight comparisons that were performed (between plant type within each treatment), five were in favour of the susceptible line (Fig. 3a). A sign test again reveals that this is consistent with a similar behaviour from both biotypes. Moreover, the pattern appeared similar whatever the treatment.

From the total length of the inflorescence

The mean and 95% confidence intervals of the total lengths of the inflorescences measured at harvest are presented in Fig. 3b as a function of treatment and plant type. As for the dry weight of the glomerules, increasing density decreased the length of the inflorescence. A comparisons of the means conducted for each treatment revealed that the susceptible plants were better at intermediate densities when grown in mixed stands. The significance level is however close to 5% for one of the two significant comparisons (Table 2).

Since the dry weight of the glomerules appeared as a good estimate of seed number, as had already been shown for total inflorescence length, the similarity of seed production in the two biotypes could safely be inferred from the above results.

Seed germination

Figure 4 presents the germination curves for the two biotypes. No effect of the mother plant was found and all seeds were pooled within each biotype. The final germination rate and the speed of germination were similar for the two biotypes (for resistant and susceptible respectively, germination rates: 0.707±0.087 and 0.758±0.095, $P>0.05$; number of days for germination: 98.2±10.9 and 102.8±10.2).

Discussion

A simplified view of evolution would be that the genes which raise the fitness of the individuals that carry them are likely to increase in frequency, subject to developmental, mechanical and genetical constraints. Therefore, evaluating the extent to which a herbicide resistance gene changes the fitness of an individual provides an indication of whether this resistance is likely to spread in wild populations and whether it will be eliminated if no herbicide is applied. For this reason comparing the fitness of resistant and susceptible plants is an obligate step in risk-assessment studies of their deliberate release.

The herbicide-resistant line of chicory described in the present paper is not transgenic. In terms of risks assessment, however, it is the phenotype of the plant rather than the technique used to produce it that should be considered (Tiedje et al. 1989). Most of the risks associated with the commercial release of a herbicide-resistant crop, namely the spread of the resistance in wild/weed related species and the development of new weeds if the resistant crop persists after harvest, are indeed largely independent of the technique used to introduce the resistance. Possible changes in the fitness of the plants were therefore considered.

Measuring the fitness of an individual is a difficult task since fitness depends not only on straitforward traits such as seed production and germination but also on more complex life-history traits including phenotypic plasticity and trade-offs between traits (e.g. Schmid 1992; Stearns 1992). It is also dependent on environmental conditions, especially density, i.e. competition (Reboud and Till-Bottraud 1991). Comparing genetically homogeneous lines however makes things easier because traits not affected by the resistance are likely to be similar when the plants are grown in the same environment.

During the first year of the experiment, we chose to estimate the vegetative biomass of the two lines because this character is believed to be correlated with competitive ability and overwinter survival. Our results, based on the number of leaves and plant height indicate that the two lines behaved in a similar way.

The reproductive characteristics of the two lines were compared during the second year. The traits we opted for were seed production and germination and, also, pollen production and efficiency because they determine the number of offspring originating from the female and the male functions respectively. Furthermore seeds and pollen are the two main vectors by which the resistance gene could escape from the field into wild populations. No significant difference between the two lines were observed in any of the measured traits at any density.

These results are consistent with those found by Mallory-Smith et al. (1992) and Thompson and Thill (1992) on *K. scoparia* in that the resistant individuals expressed a very high level of resistance together with a fitness similar to that of the susceptible ones when no herbicide was sprayed. This is also consistent with the results of Swanson et al. (1989) on a mutant *Zea mays*. The other sulfonylurea-resistant crops on which such studies were carried out (McHugen and Holm 1991; Brandle and Miki 1993) are transgenic so that fitness differences might be due to the site of gene insertion and cannot be directly compared with our present results.

Apart from the resistance, another difference between the two lines we used is that the resistant one originated from tissue culture whereas the susceptible one did not. Plant regeneration from cultured tissues and cells is known to result in genetic variation among regenerated plants (Larkin and Scowcroft 1981; Dale and McPartlan 1992). Some unknown genetic differences might therefore have existed between our susceptible and resistant lines, though no morphological changes were apparent. If we had found that the two lines differed in field performances, it would have proved difficult to discriminate between the consequences of tissue culture and those of the resistance. This was not the case and, as it is most improbable that these two effects compensate for each other in the resistant line, we can safely conclude that the tissue culture and the resistance did not affect the behaviour of the resistant line. Moreover, this study allowed us to compare an actual cultivated line with a resistant line that could be commercialised.

In terms of ecological-risk assessment of the deliberate release of this crop, two possibilities should be considered regarding the spread of the resistance: (1) the crop itself becomes a weed, (2) the resistance gene integrates in a population of a wild species through hybridisation, thus creating a new weed.

The only data available regarding the spread of sulfonylurea-resistant plants are those concerning *K. scoparia*, *L. serriola*, *Stellaria media* and *Salsola iberica* (Thill et al. 1991). These species became major weed problems in fields after 3–5 years of herbicide treatment. They cannot, however, stand as direct examples for what will happen with the escape of resistant crops into wild populations because (1) they are continuously favoured by the herbicide treatments in the field and (2) they already possessed the biological characteristics that made them a weed. By contrast, (1) wild plant populations will probably not be treated with herbicide outside of the field and (2) most crop plants are often uncompetitive outside of the field (Harlan 1975). The latter might, however, not be true for crop plants such as alfalfa or rapeseed which might be able to establish themselves outside the fields in western countries (Jacot and Jacot 1994). Since our results show that resistant and susceptible chicories have a similar fitness, it is highly unlikely that chicory could become a problem in the absence of herbicide treatments with sulfonylurea.

Escape of the resistance gene itself from the crop to wild relatives via pollen is likely to happen in chicory since the wild and the cultivated biotypes belong to the same species and can cross easily. As (1) the genetic background in the hybrids will differ from that of the crop and (2) the resistance gene will be transferred in the hybrids with genetic material from the crop, conclusions on the effect of the resistance gene should be drawn with considerable caution. It can however be suggested that the mutant acetolactate synthase will not decrease the fitness of the hybrids. If this is the case, the resistance gene should remain in these populations of wild chicory even in the absence of herbicide treatment and thus form a reservoir of resistance genes. Whether this would cause ecological or agricultural problems will depend mainly on the gene flow from resistant crops, the occurrence of fields treated with sulfonylureas and the frequency of wild chicory in and outside these fields.

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