Burial and seed survival in *Brassica napus* subsp. *oleifera* and *Sinapis arvensis* including a comparison of transgenic and non-transgenic lines of the crop

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SUMMARY

The creation of transgenic plants through genetic engineering has focused interest on how the fitness of a plant species may be altered by small changes in its genome. This study concentrates on a key component of fitness: persistence of seeds overwinter. Seeds of three lines of oilseed rape (*Brassica napus* subsp. *oleifera* DC Metzger) and of charlock (*Sinapis arvensis* L.) were buried in nylon mesh bags at two depths in four habitats in each of three geographically separated sites: Cornwall, Berkshire and Sutherland. Seeds were recovered after 12 and 24 months. Charlock exhibited much greater seed survival (average 60% surviving the first year and 32.5% surviving the second year) than oilseed rape (1.5% surviving the first year and 0.2% surviving the second) at all sites. Charlock showed higher survival at 15 cm burial than 2 cm burial at certain sites, but oilseed rape showed no depth effect. Different genetic lines of oilseed rape displayed different rates of seed survival; non-transgenic rape showed greater survival (2%) than the two transgenic lines, one developed for tolerance to the antibiotic kanamycin (0.3%) and one for tolerance to both kanamycin and the herbicide glufosinate (0.25%). The absolute and relative performances of the different genetic lines of oilseed rape were context specific, illustrating the need to test hypotheses in a wide range of ecological settings.

1. INTRODUCTION

Advances in recombinant DNA technology have resulted in the production of a new generation of crop plants containing a variety of traits, e.g. herbicide resistance, disease and pest resistance, and tolerance to environmental stress. Before they are released on a wide scale, it is vital that any risks which may be attached to their use are assessed on sound scientific principles (Regal 1989). There has been some debate as to whether the process of genetic engineering may be considered merely as an extension of selective breeding (Teidje *et al*. 1989), or whether it has the potential to be profoundly different (Regal 1994). When a host plant is provided with novel features, not available in the gene pool of compatible species, which may increase its competitiveness, the latter conservative approach may be more appropriate.

One of the most common attributes to be engineered into crops in the early days of this technology is herbicide resistance. It is not expected that this feature would increase a plant's competitiveness in the absence of selection pressure (i.e. the herbicide), although there is always the possible 'wild card' of pleiotropic effects (Regal 1994). Nevertheless, herbicide-resistant transgenics are the subject of some concern to environmentalists and ecologists alike as it is not clear whether these constructs would promote the use of environmentally benign herbicides, potentially reducing the net use of agrochemicals, or result in the overuse of certain herbicides (Dyer 1994). Another major consideration is that the herbicide resistance genes may escape into the seed bank, either in the seeds of weedy relatives, or in persistent seeds from the crop itself (Goodman 1987; Dyer 1994). Over a period of time, and with the introduction of different transgenic crops, the seed bank might accumulate genes conferring resistance to a number of herbicides. Such populations could be costly to control, and the genes could spread to other closely related species (Dale 1992; Adler *et al*. 1993; Scheffler & Dale 1994).

It is well known that viable seeds of oilseed rape can persist in arable soils for up to 10 years or more (Vaughan *et al*. 1976), and that volunteer oilseed rape can be a serious weed of subsequent broad-leaved crops (Ward *et al*. 1985). The ability to persist in a dormant state for such long periods undoubtedly contributes to the success of *B. napus* in colonizing disturbed habitats, and therefore factors which alter persistence are likely to influence fitness.

Here we describe an experiment on the comparative survival of three genetic lines of spring oilseed rape (one conventional and two transgenics). One non-transgenic line of a weedy relative, charlock, which is known to exhibit protracted seed dormancy (Wilson

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1987) was included as a control, to assess the relative preservative properties of the different soils at our twelve study sites. Informed expectation would be that any additional genetic material which provides a benefit (e.g. herbicide tolerance) will also necessarily carry a cost (e.g. a metabolic cost). In the absence of selection pressure which causes the transgenic plant to be favoured, the additional cost of metabolizing the detoxifier could cause the transgenic to be less competitive then the wild type. This has been referred to as the genetic baggage hypothesis (Regal 1988). If, however, in the absence of selection pressure, the gene is not expressed, then the transgenic may not carry significant genetic baggage. However, other considerations make it unlikely that the carriage of extra genetic material would be cost-free – for example, the position in which the genes had been integrated into the genome cannot be controlled, and the resulting disruption could well lead to associated costs. However, in other studies in which marker genes have been inserted into plants, no measurable differences in the fitness of the transgenics have been detected. One such example is the inclusion of a kanamycin-tolerance gene (the same as is used in this study, and whose function is to allow the selection of cells during the transformation process) into potato plants (Dale & McPartlan 1992). It would appear, therefore, that many insertion sites do not cause measurable disruption. Of greater concern is the possibility that some traits may, purposefully or inadvertently, result in features which will be of selective advantage in particular habitats (e.g. increased tolerance to extreme environmental conditions). Furthermore, ecological performance is highly context specific: the same genotype will give rise to phenotypes with different fitnesses in different environments (e.g. Crawley 1990; Rees *et al*. 1991). It is not possible to predict precisely which outcome will apply to which transgenic crop in which habitat. Yet there are almost no empirical data available to tackle this issue for any transgenic crop (Wrubel *et al*. 1992).

2. METHODS AND MATERIALS

(*a*) *Experimental material*

The species chosen for this study was oilseed rape, *Brassica napus* subsp. *oleifera*, a member of the family Cruciferae. It has both annual and biennial forms: var *biennis* is grown as winter rape, whilst var *annua* is grown as spring rape. It reproduces only by seed, having no vegetative means of reproduction or long-term perennation such as tubers or rhizomes. (However, it has been found that a small proportion of heavily grazed plants may persist for an extra winter and set seed the following year: Hails *et al*. unpublished data). Spring rape is always semelparous, whilst winter rape usually is. A spring cultivar, Westar, was used in this study for both conventional and transgenic lines. There were two transgenic lines: the first containing resistance to the antibiotic kanamycin, and the second containing both kanamycin resistance and resistance to the herbicide glufosinate. The expression of the kanamycin resistance gene is used to select transformed cells in the laboratory by growing them on a medium containing kanamycin. It is therefore a marker gene, the expression of which is not designed to confer any selective advantage to the transformed plant in the field. The herbicide glufosinate (marketed as Basta in Europe and Challenge in the UK) is a non-selective, partially systemic, contact herbicide which acts by inhibiting the detoxification of ammonia.

The three genetic lines may therefore be summarized as control, kanamycin-tolerant and kanamycin-with-glufosinate tolerance (hereafter referred to as glufosinate). The genetic transformation process was initially carried out on a few cells, which were then regenerated into whole plants. These plants were then taken through several generations (by selfing) to produce sufficient seed for a large scale field trial. Some of these plants were homozygous, some were heterozygous for the transformed gene(s), and some did not possess the introduced genes at all. A sample from the same stock of herbicide-tolerant seeds was found to contain 65 % of transgenic seeds, 95 % of these being homozygous for the herbicide tolerant gene (Scheffler *et al*. 1993).

A close relative, known to exhibit high persistence in many habitats and generally regarded as a weed, is charlock (*Sinapis arvensis*). Seeds of this species were included in the experimental design to provide a standard against which the persistence of oilseed rape could be assessed, and to demonstrate the extent to which habitats were inimical to seed survival in general.

(*b*) *Study sites*

The study sites were chosen to be in three different climatic zones, and in a range of habitats within each zone. The first zone represented a benign climate with an early start to the growing season (Cornwall, in extreme south-western England), the second an intermediate, more continental climate (Berkshire, in south-central England, where the growing season begins approximately 4 weeks later than Cornwall), and the third, a more hostile climate with a late start to the growing season (Sutherland in north-east Scotland, where the season begins about 8 weeks later than in Cornwall). A brief description of the 12 habitats is given in table 1.

(*c*) *Experimental design*

The experiment was conducted within four blocks (25 m by 25 m) fenced against rabbits and deer, located at random within each habitat, and formed part of a wider study of the ecology and invasive potential of oilseed rape in natural habitats (Crawley *et al*. 1993). Within each block, the experiment was laid out in 16 quadrats (two depths, three genetic lines plus *S. arvensis*, 2 years of retrieval). Each treatment combination appeared in every block. Cultivation (i.e. disturbance) was carried out once at the initiation of the experiment.

Seeds were encased in an envelope of 1 mm² nylon mesh, fine enough to retain the seeds, yet coarse enough to allow seedlings to germinate and grow. This allowed the seeds to be retrieved after 1 and 2 years in the soil. Each nylon envelope contained 50 seeds and was buried within a quadrat at one of two depths (2 cm for shallow burial and 15 cm for deeper burial). Each quadrat was marked by individually numbered metal tags. After retrieval, each seed was examined and dissected to determine its fate. Seeds with a split testa were recorded as having germinated, those with firm, pale cotyledons were recorded as viable and those with soft, discoloured cotyledons were recorded as dead. Preliminary tests with tetrazolium dye (Porter *et al*. 1947; Rees & Brown 1991) indicated that this method satisfactorily determined a seed's state.

Table 1. *The* 12 *habitats*

habitat code	Cornwall	habitat code	Berkshire	habitat code	Sutherland
H1	*Penkestle Down* GR 20/140645 Lowland grassland	H5	*Merten's Acres* GR 41/946690 Naturally regenerated oak woodland	H9	*Camore Wood* Sheltered, sandy quarry in a Scots pine woodland
H2	*Great Wood, Tregays* GR 20/125560 Lowland, estuarine oak woodland	H6	*Gunness's Hill* GR 41/944687 Bracken on a sandy soil	H10	*Achormlarie Peat* GR 82/695957 Exposed, acid peatland
H3	*Great Grogley Downs* GR 20/015675 Middle elevation health community	H7	*Pound Hill* GR 41/937693 Disused arable field	H11	*Achormlarie Mineral* GR 82/7697946 Well drained area of acid grassland and bracken
H4	*Davidstow Woods* GR 20/152842 Acid grassland surrounded by a conifer plantation	H8	*Rush Meadow* GR 41/938691 Wet grassland, dominated by rushes, grasses and wetland herbs	H12	*Sallachy* GR 92/559067 Open, naturally regenerated birch woodland

(*d*) *Statistical analysis*

The response variable of interest was the number of seeds that remain viable after differing lengths of time in the soil. The data were first analysed separately for each habitat (and later combined where possible), and fitting the explanatory factors (genetic line, depth of burial and year). Several different numerical transformations and error structures were used, and the one which best represented the data was chosen. In all cases, the analysis was weighted for the number of seeds that were estimated to be viable at the beginning of the year in question. All models were simplified to produce a minimal adequate model (details in Crawley 1993). A set of orthogonal contrasts was made including: (1) a comparison of *S. arvensis* with oilseed rape; (2) a comparison of the conventional with the transgenic lines of oilseed rape; (3) a comparison of the two transgenic lines.

3. RESULTS

(*a*) *Variability between habitats*

The percentage of seeds remaining dormant differed considerably between habitats (as illustrated in figure 1 for *S. arvensis*). Whilst habitats within the Berkshire site were fairly similar, there were more significant differences between the habitats at the Cornwall and Sutherland sites.

(*b*) *Variability between genetic lines*

In spite of the between-habitat variability, the weedy *S. arvensis* always showed higher seed survival than the crop plant *B. napus* (table 2, Baker 1972). Even in the most inhospitable habitat (Sutherland, H9, Camore Wood), *S. arvensis* was significantly more persistent than any of the three genetic lines of oilseed rape ($F_{1,36} = 519.2$, $p < 0.001$; ANOVA with contrasts between species, square root transform with normal errors).

Oilseed rape has been bred for immediate germination of seeds, as this is obviously desirable in an agricultural context. Of all *B. napus* seeds used in the experiment, only 1.5 % persisted beyond the first year,

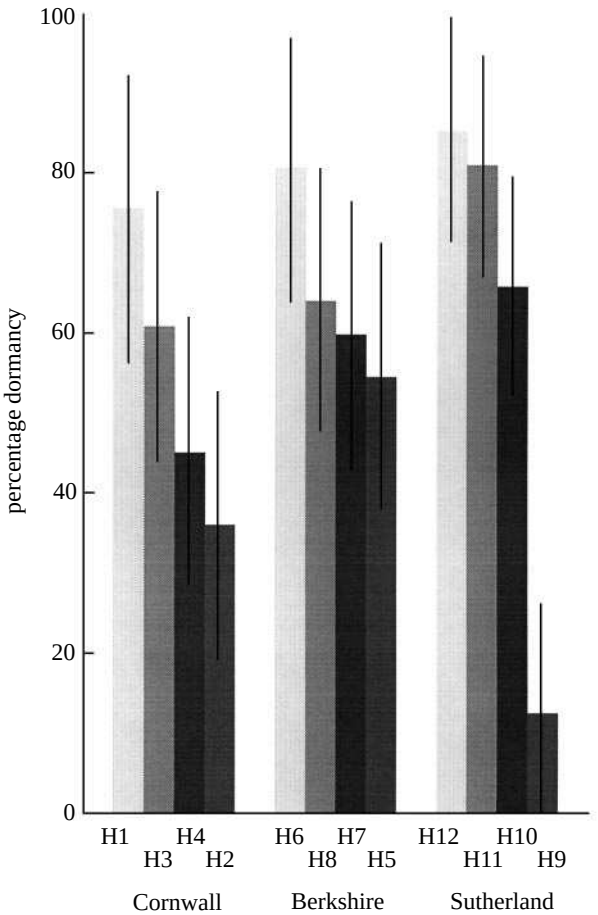

Figure 1. The percentage of *S. arvensis* seeds remaining dormant after 12 months burial in each of the 12 habitats. These figures are averaged over two depths. Error bars are pooled standard errors for that site converted to LSD bars for graphical purposes.

Table 2. *The total number of seeds* (*out of 400*) *viable after burial for 12 and 24 months, for three genetic lines of* Brassica napus (*osr*) *and a wild relative* S. arvensis

(These figures have been summed over two depths (2 cm and 15 cm) within each habitat. The habitat codes correspond to the habitats described in table 1.)

		Sinapis		control osr		kanamycin osr		glufosinate osr	
		year 1	year 2	year 1	year 2	year 1	year 2	year 1	year 2
Cornwall	H1	302	267	60	6	1	0	1	0
	H2	144	29	3	0	2	1	0	0
	H3	243	79	5	0	2	0	0	0
	H4	180	17	0	0	0	0	4	0
Berkshire	H5	218	134	37	2	4	0	10	0
	H6	322	71	16	1	11	0	0	0
	H7	239	232	0	1	0	0	1	0
	H8	256	141	1	0	0	0	1	0
Sutherland	H9	50	2	0	9	0	1	1	0
	H10	263	12	28	0	2	0	1	0
	H11	324	229	16	5	6	2	0	0
	H12	341	352	6	0	0	1	2	0
mean (per genetic line per year)		240	130	14	2	2	0.4	2	0

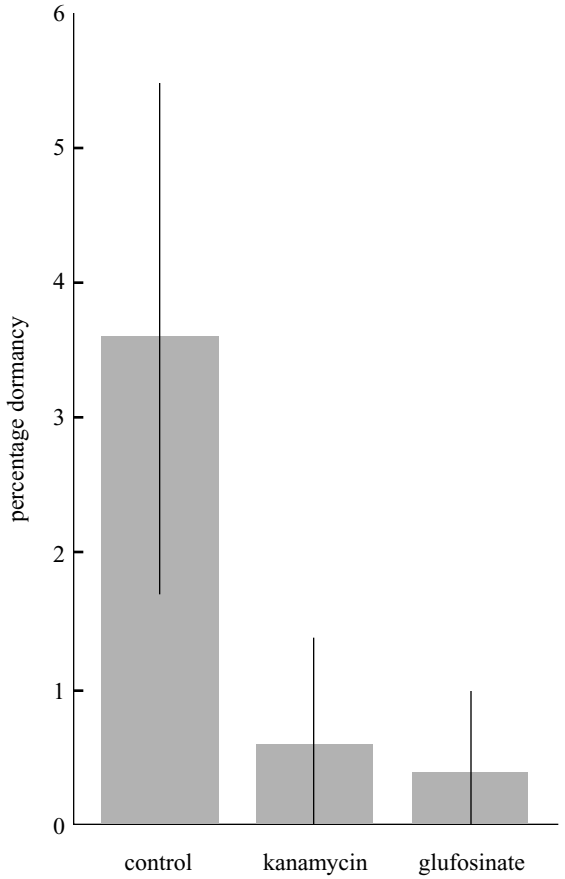

Figure 2. The percentage of *B. napus* seeds remaining dormant after 1 year of burial for three genetic lines. These means are averaged over two depths and 12 habitats. Error bars are standard errors of the means. In five of the 12 habitats, a significantly greater number of control seeds survived (Cornwall: H1, $F_{1,37} = 20.2$, $p < 0.001$; Berkshire: H5, $F_{1,37} = 5.86$, $p < 0.05$; H6, $F_{1,28} = 8.09$, $p < 0.05$; Sutherland: H10, $F_{1,28} = 24.9$, $p < 0.001$, H11, $F_{1,17} = 4.96$, $p < 0.05$; in all cases ANOVA with contrasts between control and transgenic genetic lines and a square root transform with normal errors).

but a significantly greater number of these seeds were control rather than transgenic seeds (figure 2; statistics in the figure legend).

However, when the data were examined separately for each habitat, this response was only detectable in five habitats. In the other seven habitats, there was no detectable difference in the behaviour of control and transgenic seeds, as the vast majority had germinated in year one.

(*c*) *Dormancy and depth of burial*

Depth of burial increases the probability of an *S. arvensis* seed remaining dormant (0.58 at 2 cm and 0.72 at 15 cm after one year of burial; mean probability averaged over 12 habitats). When analysed by habitat it was only significant in four of the 12 habitats. In each case there was a significant interaction between species and depth, indicating that whilst depth increased the probability of remaining dormant for *S. arvensis*, it did not do so for *B. napus* (Cornwall H1: $F_{1,60} = 21.5$, $p < 0.001$; Cornwall H2: $F_{1,57} = 12.9$, $p < 0.001$,; Berkshire H6: $F_{1,57} = 4.82$, $p < 0.05$; Berkshire H7: $F_{1,60} = 18.0$, $p < 0.001$; ANOVA with normal errors in all cases). The lack of pattern with depth for oilseed rape seeds is probably due to dormant seeds being so few in number for this species, even when all three genetic lines were pooled.

(*d*) *Comparing the two transgenic genotypes*

Of all transgenic seeds used in the experiment, 98% germinated (based on a split testa observed on dissection). Fewer seeds of the genetic line carrying both the glufosinate and kanamycin tolerance genes survived year 1 compared to the line carrying only kanamycin tolerance (28 versus 21), but this difference was not significant ($\chi_1^2 = 1.0$, n.s.).

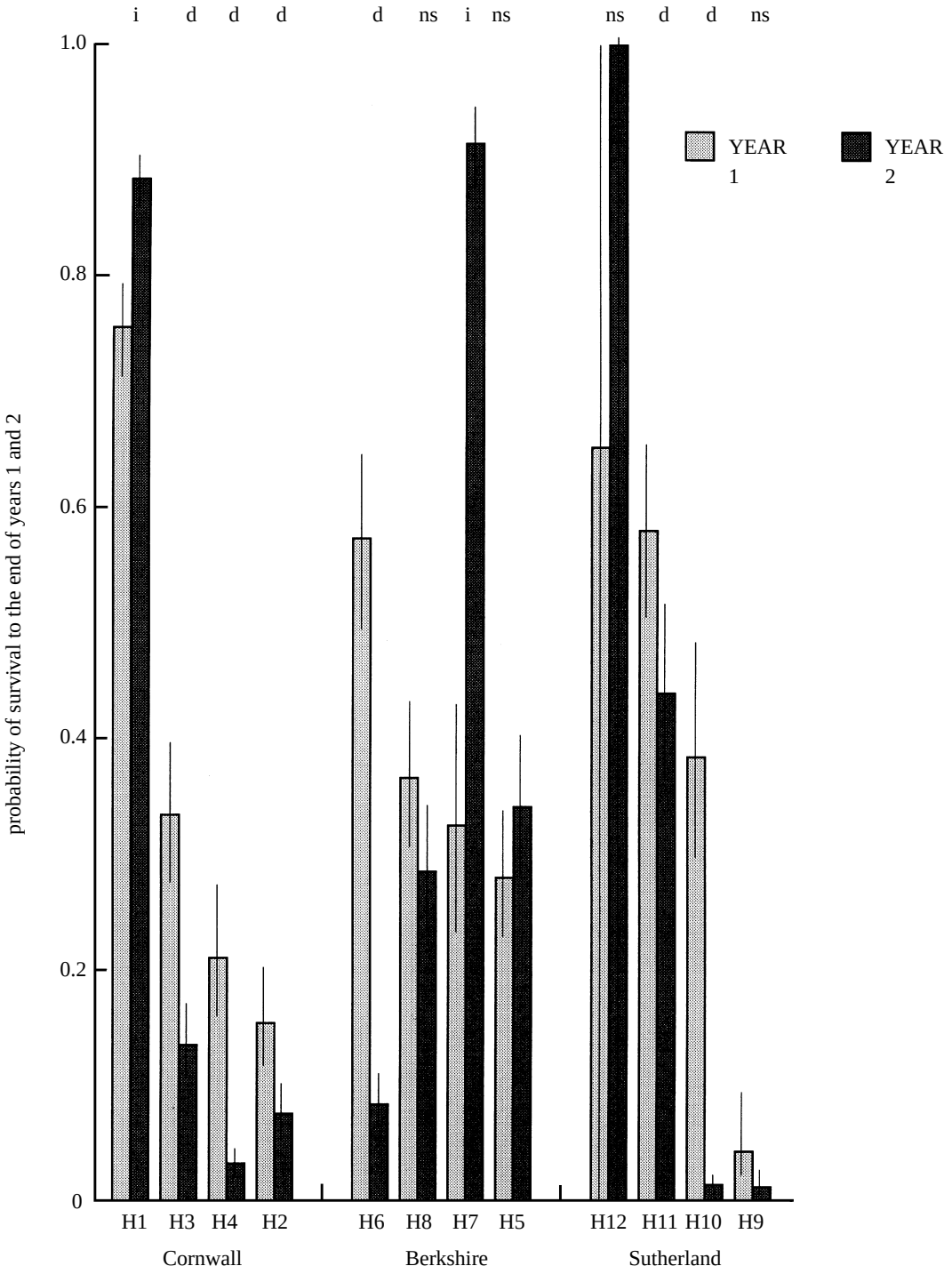

Figure 3. The probability of surviving until the end of the year for *S. arvensis* seeds in years 1 and 2 of burial. Error bars are LSD bars for the difference between years 1 and 2 (obtained from the binomial model and backtransformed), and therefore act as a visual *t*-test between the 2 years for that habitat. In six habitats, the probability of survival in year 2 was significantly lower than in year 1 (those pairwise comparisons with a *d* above); in two habitats there was a significant increase (denoted by *i*), and in the other four, the difference was not significant.

When analysed per habitat, and weighted according to the number of seeds present at the beginning of the year in question, the probability of survival in the seed's second year is significantly less for (1) H2, $F_{1,39} = 20.1$, $p < 0.001$; (2) H3, $F_{1,41} = 19.6$, $p < 0.001$; (3) H4, $F_{1,39} = 28.2$, $p < 0.001$; (4) H6, $F_{1,51} = 79.1$, $p < 0.001$; (5) H10, $F_{1,51} = 85.0$, $p < 0.001$; (6) H11, $F_{1,40} = 5.12$, $p < 0.05$; all ANOVA with normal errors and a square root transform.

(*e*) *Age dependent seed survival in* S. arvensis

The number of seeds which survived until the end of year 1 provided an estimate of the numbers entering the second year. Thus the proportion of seeds surviving a second year of burial was analysed using this estimate as a binomial denominator. This revealed that in six of the 12 habitats, the probability of survival was significantly reduced in the second year (figure 3). The fate of their *S. arvensis* seeds was therefore dependent on its age.

Seed survival beyond year 1 was so low for all three

B. napus lines that no age-dependent effects on the fate of oilseed rape seeds were detected.

4. DISCUSSION

The behaviour of seeds from all three genetic lines of *B. napus* and *S. arvensis* was shown to be highly context specific. There was great variability in their persistence from habitat to habitat (with, for example, only 12.5 % survival for *S. arvensis* seeds in Sutherland H9, but 85.3 % survival in Sutherland H12). Similarly, whilst there was a significant difference in the survival of the control compared to the transgenic *B. napus* lines, this was only significant in just under half the habitats (five), though in no habitat was this trend reversed. It may be that this latter response of genetic line was difficult to detect due to the very low rates of survival of oilseed rape seed, or it may be that this differential persistence only occurred in some environments. Similarly, whilst *S. arvensis* seed exhibited better survival at the greater depth, this response was only significant in four of the 12 habitats. This illustrates the importance of testing hypotheses in a variety of ecological contexts.

Whilst there were differences between the control and transgenic lines, no differences were detected between the two transgenic lines, in spite of the fact that one carried genetic material for two additional traits (glufosinate and kanamycin tolerance) compared to one (kanamycin tolerance alone). This latter comparison, therefore, does not provide any support for the genetic baggage hypothesis. However, it would be naive to imagine that the cost of extra genetic material would be simply additive. There are a number of other considerations to include when comparing both the control and transgenic lines, as well as the two transgenics. First, we are left with the question of whether it was the genotype itself or the process by which it was constructed that led to the reduced persistence of the two transgenic lines. When plants are regenerated from cells in tissue culture, intense selection pressure is placed on those cells in an environment in which most cells are inhibited from dividing by the antibiotic kanamycin. Only those cells containing the kanamycin tolerance gene can survive. At this point, genetic variation of the regenerated plants often results (somaclonal variation). The degree and nature of this variation is likely to vary between the two transgenic groups, and in other studies, such variation has been found to influence plant performance (Dale & McPartlan 1992).

Second, the number of copies of the inserted genes may differ between the two transgenic groups; for example, it is conceivable that two copies of the kanamycin tolerance gene were incorporated into the kanamycin-tolerant line, but only one of the kanamycin and glufosinate genes in the herbicide-tolerant transgenic line. The method used (*Agrobacterium* method) has been observed to result in more than one copy (Dale *et al.* 1993). However, DNA hybridization analysis was used to choose transformants containing a single insert, therefore this is unlikely to have been a factor in this study. It is more likely that the two transgenic groups may have differed in their levels of zygosity of the transgenes after multiplication through selfing. Thirdly, the position of insertion of the foreign genes into the genome is not controlled, and therefore could disrupt genome function (insertion mutagenesis). Only those cells in which cell function has not been disrupted in a major way will survive to regenerate into plants. However, different positions of insertion of the transgene(s) on the genome might result in variable degrees of disruption that could, in turn, result in variation in cost to the plant. It is quite possible that the extra costs of expressing a herbicide resistance gene could be masked by the fact that the kanamycin-only construct had incorporated its foreign DNA in a more disruptive position.

In over half of the habitats, the probability of seed survival in *S. arvensis* was age-dependent. *S. arvensis* has been shown to have complex patterns of recruitment (Rees & Long 1993) which are consistent with the age-dependent survival found here. In two habitats there was a significant increase in the probability of survival in the second year, whilst in six habitats there was a significant decrease. It was assumed that all seeds entering year 1 were viable, yet clearly that may not have been the case. Therefore, the expectation would be that survival would be higher in the second year, as the non-viable fraction would have been accounted for as mortality in the first year. However, this expectation was not borne out, and the data illustrate the reverse trend in a significant number of cases (binomial $p < 0.0001$). Simple models illustrating the mechanism by which dormancy may evolve often assume age-independent probability of survival (Cohen 1966). This decrease in survival represents a cost to persistence for the seed, and would therefore act to reduce selection pressure on the evolution of dormancy.

To resolve ecological concerns about transgenic plants, it is necessary to expand the current database on the behaviour of these plants, not only in small agricultural plots but more importantly in natural habitats. These data provide information on part of the broader picture, namely persistence of buried seeds of the transgenic crop. This is only one component of fitness; other components include survival and reproduction (Crawley *et al.* 1993). Whilst this study illustrates that for these constructs there is no enhanced risk that the transgenic seeds will persist, this is not the only potential reservoir for the transgenes that should be considered. It has recently been demonstrated that transgenes from oilseed rape can appear in the weedy relative *B. campestris* in the first back-cross generation (Mikkelsen *et al.* 1996), and the persistence of these hybrid seeds will be of crucial importance in determining the long-term dynamics of the transgenes.

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