Fitness costs associated with transgenic glufosinate tolerance introgressed from *Brassica napus* ssp *oleifera* (oilseed rape) into weedy *Brassica rapa*

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ABSTRACT

Wild relatives of genetically engineered crops can acquire transgenic traits such as herbicide tolerance *via* spontaneous crop-wild hybridisation. The fitness costs associated with a transgene conferring tolerance of glufosinate were tested when introgressed into weedy *Brassica rapa*. Crosses were made between transgenic *Brassica napus* ssp *oleifera* (oilseed rape) and wild *B. rapa* from Denmark. The reproductive success of 457 BC_3 progeny representing six full-sib families raised in growth rooms was quantified (plants were pollinated by captive bumblebees). Segregation for herbicide tolerance was 1:1 overall, as expected for a dominant Mendelian trait. There were no significant differences between transgenic and non-transgenic plants in survival or the number of seeds per plant, indicating that fitness costs associated with the transgene are likely to be negligible. Pollen fertility and seed production of BC_3 plants were as great as those of *B. rapa* raised in the same growth rooms. These results suggest that transgenic herbicide tolerance is capable of introgressing into populations of *B. rapa* and persisting, even in the absence of selection due to applications of this herbicide.

INTRODUCTION

A widely acknowledged drawback of genetically engineered crops is the potential for transgenes to spread to related weeds *via* crop-weed hybridisation (e.g. Ellstrand & Hoffman, 1992; Raybould & Gray, 1993). Crops that are known to interbreed with weedy relatives under field conditions include rice, sorghum, squash, oilseed rape (canola), sunflower, sugar beet, carrot, strawberry, radish, wheat, oats, and others (Snow & Morán Palma, 1997; Zemetra *et al.*, 1997). Most of these economically important crops have been targeted for improvement by the addition of transgenic traits and several have already been released commercially, including herbicide tolerant oilseed rape. In the short term, the most obvious problem cases will likely be weeds that acquire transgenic tolerance of widely used, broad-spectrum herbicides such as glyphosate and glufosinate (Dyer, 1994). With the exception of *Lolium rigidum* in Australia (Powles *et al.*, 1998), repeated use of these particular herbicides has not yet led to the evolution of tolerant weed genotypes (Bradshaw *et al.*, 1997; Heap, 1997), as often occurs with other types of herbicides. Now, however, weedy taxa that hybridise with transgenic crops will be able to acquire tolerance of glyphosate and glufosinate following crop-to-wild gene flow.

Once transgenes conferring herbicide tolerance move into weeds, their frequency within local populations will be influenced by; 1) continued immigration due to gene flow from cultivated plants; 2) strong selection favouring herbicide-tolerant genotypes during periods of exposure to the herbicide; 3) in the absence of the herbicide, the possibility of selection *against* these genotypes if the transgene is associated with reduced fitness. Such costs could be caused by pleiotropy, physiological costs of the tolerance trait or effects of particular insertion sites within the genome (such as linkage to deleterious alleles or disruption of coding regions; Bergelson *et al.*, 1996). In commercialised transgenic crops, the authors expect that any costs of herbicide tolerance will be minimal due to economic incentives for developing high-yielding, herbicide-tolerant varieties. These costs could be different in weeds than in crops, however, due to factors such as different genetic backgrounds, genotype-by-environment interactions or crop genes that are tightly linked to the transgene insertion site and are deleterious to the weed but not the crop.

The purpose of this study was to test for fitness costs associated with transgenic tolerance of glufosinate in a widespread weed, *Brassica rapa* L. (= *B. campestris*), which hybridises with oilseed rape. The study focused on glufosinate tolerant oilseed rape as one of the first crops that could affect the abundance of an agricultural weed (as a result of crop-wild hybridisation followed by the use of this type of herbicide). A secondary goal of the study was to characterise the seed production of selected backcrossed plants (the BC_3 generation with *B. rapa* as the recurrent parent) as compared to the seed production of pure *B. rapa* in order to detect major abnormalities, if any, resulting from introgression. A more detailed description of the study can be found in Snow *et al.* (1999).

Wild *B. rapa*, commonly known as field mustard or bird rape, is an economically important weed in temperate regions of Eurasia, North America, South Africa, Australia, and New Zealand (Holm *et al.*, 1997) and is often seen in fields of oilseed rape (e.g., Jørgensen & Andersen, 1994). *Brassica napus* ssp *oleifera* (oilseed rape) is an ancient allotetraploid possessing the AA genome from *B. rapa* (2n=20) as well as the CC genome from *B. oleracea* (2n=18; Song *et al.*, 1995). Thus, F_1 hybrids between *B. napus* and *B. rapa* have a triploid AAC genome constitution of 2n=29. Because they have a full complement of the *B. rapa* genome, fertility of some F_1 progeny can be almost as high as that of pure *B. rapa* (Jørgensen & Andersen, 1994; Jørgensen *et al.*, 1996; Hauser *et al.*, 1997).

Crop-to-wild hybridisation is facilitated by the fact that *B. rapa* is an obligate outcrosser that relies on bees, other insects, and possibly wind to effect pollen transfer (Jørgensen & Andersen, 1994). Pollen from fields of *B. napus* has been detected at distances of up to 1.5 km away from the crop (e.g., Timmons *et al.*; also see Scott & Wilkinson, 1998) and further gene movement *via* human-mediated seed dispersal may be common. Spontaneous backcrossing to the weed has been demonstrated in field experiments involving both DNA markers and transgenic tolerance of glufosinate (Jørgensen *et al.*, 1996; Mikklesen *et al.*, 1996).

METHODS AND MATERIALS

Transgenic BC_2 plants were used, described in Mikkelsen *et al.* (1996), to create a BC_3 generation segregating for the presence or absence of three tightly linked transgenes: the *bar* gene for glufosinate tolerance, the *neo* gene coding for NPTII (conferring resistance to

kanamycin), and the *barstar* gene, which restores male fertility. This multiple-gene construct has no detectable effects on the yield or competitive ability of Drakkar oilseed rape (Fredshavn *et al.,* 1995). The crosses made to obtain the BC_3 generation involved varying numbers of indoor-grown plants, depending on their availability at times when crosses were conducted (see Snow *et al.*, 1999). First, *B. rapa* plants from a wild population in Denmark were crossed with transgenic *B. napus*. Transgenic F_1 progeny were then crossed with *B. rapa*, and transgenic BC_1 plants were crossed with *B. rapa*. Six BC_2 progeny were each crossed with a different *B. rapa* plant. Thus, the BC_3 progeny used in this study were divided into six full-sib families: 1-3, 1-4, 1-10, 1-12, 1-13, and 9-14, with the first number denoting the seed's "maternal grandmother" and the second number referring to the *B. rapa* paternal parent from the wild population (each full-sib family had a unique maternal and paternal parent).

To test for costs associated with glufosinate tolerance, BC_3 plants were grown that segregated for the presence or absence of glufosinate tolerance and recorded the total number of fruits and seeds produced by each plant. Progeny from each family were grown under the same environmental conditions, but due to lack of space and temporary technical problems with two growth rooms, it was not possible to put all of the families in the same growth room or to distribute them equally among rooms. Therefore, seed production differences among families cannot be distinguished from differences due to environmental conditions of the growth rooms because these two factors were confounded. All plants within each family were treated identically, however, so that effects of being transgenic were tested within each family and the entire data set was analysed by nesting families within growth rooms for analyses of variance. *B. rapa* plants from a wild population in Denmark were also grown to serve as a reference for general comparisons with the BC_3 generation.

Although light levels in the growth rooms were lower than full sunlight, the plants showed no symptoms of light deprivation and attained sizes similar to those of field-grown plants. When flowering began, a hive of bumblebees was placed in each room to allow semi-natural pollination of these obligately outcrossing plants (Biobest greenhouse pollination kits, Westerlo, Belgium). During "daylight" hours, c. 10-15 bees foraged for pollen and nectar at any given time in each room, resulting in fruit set of c. 100%. The pollen fertility was characterised by using methods described in Snow *et al.* (1999). When the plants stopped flowering, the number of fruits per plant was counted and 10 mature, undehisced, randomly selected fruits were collected from each plant for seed counts. The average number of seeds per plant was then calculated (number of fruits x average number of seeds per fruit). Transgenic tolerance of glufosinate was scored by a PCR method or by leaf dot tests (Snow *et al.*, 1999). When these two methods were compared in a sample of 74 plants, the same result was obtained in 99% of the samples (all but one), leading us to conclude that both methods give the same results.

RESULTS

Overall, 51% of the BC_3 seedlings that were herbicide-tolerant, which is very close to the expected Mendelian 50% (N= 457). The proportion of seedlings that survived to maturity and produced at least one fruit was greater than 90% in all families and was not affected by whether the plant was transgenic (Chi-square tests). Survivorship of pure *B. rapa* was >95%. Analysis of variance showed that the total number of seeds per plant was affected by family

and growth room, but was not influenced by whether the plants were transgenic (Figure 1; Snow *et al.*, 1999). Therefore, based on this measure of overall performance, no cost of transgenic tolerance of glufosinate was detected. Transgenic BC_3 plants produced fewer fruits per plant than non-transgenic plants in two of the six families (Figure 1), but the authors regard total seed output to be a more relevant component of overall fitness than fruit number. A nested ANOVA showed that neither trangenes (+ or -) nor the transgene-by-family interaction had a significant effect on the number of seeds produced per plant.

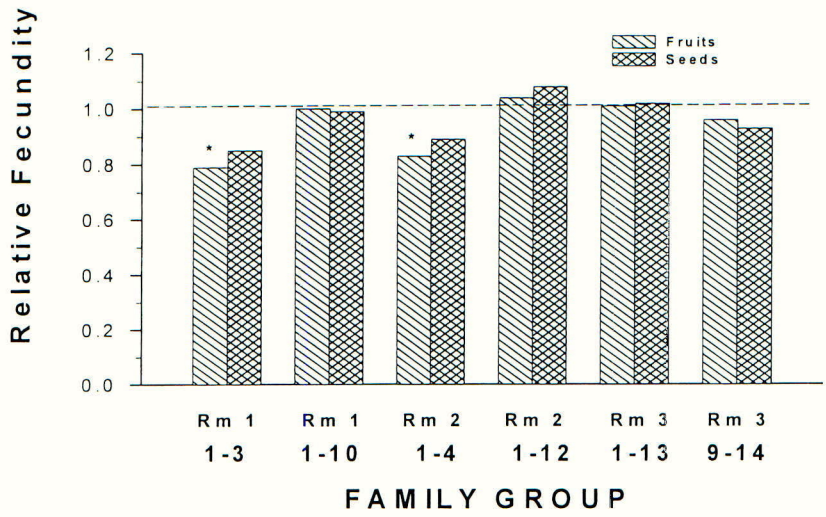

Figure 1. Relative lifetime fecundity of transgenic BC_3 progeny, calculated as the average number of seeds or fruits of transgenic plants divided by the corresponding mean for non-transgenic plants. Dotted line illustrates the expectation for equal fecundity. T-tests were used to compare fruit or seed production of transgenic *versus* non-transgenic plants within families; sample sizes were 29-51 per treatment within each family; * indicates $P<0.05$.

In comparing the fecundity of the BC_3 generation with that of pure *B. rapa*, it is important to note that the latter represent a more diverse mixture of genotypes and may have differed from the relatively small group of *B. rapa* plants involved in these crosses. Pollen fertility of plants in the BC_3 families averaged 88 - 95% and was not significantly different from the pollen fertility of *B. rapa* (92%, ANOVA, details not shown). The mean number of seeds per plant varied among BC_3 families and was higher than or similar to that of *B. rapa* plants from the same growth room (Snow *et al.*, 1999). Based on these comparisons, it is concluded that the lifetime fecundity of BC_3 plants was similar to that of a mixture of pure *B. rapa* genotypes.

DISCUSSION

This study confirmed that transgenic herbicide tolerance was transmitted to the BC_3 generation at an average frequency of c. 50%, as expected for a dominant Mendelian trait. In general, costs associated with transgenic glufosinate tolerance were small or undetectable. It is concluded that under the conditions of this experiment, the costs associated with transgenic herbicide tolerance were negligible. It would be useful to carry out further studies in the field, with more plant families and several levels of competition, to determine whether any costs of transgenic glufosinate tolerance can be detected under natural conditions.

A secondary goal was to compare the survival, pollen fertility, and seed production of BC_3 plants with those of pure *B. rapa*. BC_1 plants with >95% pollen fertility and 20-21 chromosomes were intentionally selected for the subsequent crosses in order to utilise the types of individuals that are most likely to succeed in passing crop genes on to successive generations. Thus, as expected, the BC_3 generation also had high pollen fertility and high survival. One surprising result was that the seed output of these BC_3 families was similar to or greater than the seed production of pure *B. rapa* grown under the same conditions. If this pattern also holds for plants in the field, it suggests that after crop alleles have introgressed into *B. rapa* for a few generations, those that are not strongly deleterious and are not linked to other deleterious traits would be able to persist for many subsequent generations.

Although the conclusions are limited to a particular transgenic line of oilseed rape, the results support the widespread assumption that once a transgenic trait such as glufosinate tolerance has been selected for marketing, it is not likely to entail a strong fitness disadvantage when transferred to weedy *B. rapa* populations. Clearly, there is some urgency for knowing more about this particular weed-crop complex because *B. rapa* is already a serious weed of more than 20 crops in more than 50 countries (Holm *et al.*, 1997) and glufosinate-tolerant oilseed rape is now being grown commercially. If further field experiments also demonstrate little or no costs associated with transgenic tolerance of glufosinate, it is possible that the spread of this transgene to natural populations of *B. rapa* will lead to infestations of this species that are more difficult to control. This will be especially significant in fields of transgenic oilseed rape where glufosinate applications are sometimes used as a primary control method.

In the near future, it is expected that crops such as oilseed rape will possess additional fitness-related transgenes such as those conferring tolerance of lepidopteran pests, fungal diseases, and other types of herbicides such as glyphosate. Further research on ecological implications for weedy relatives should focus on the benefits as well as possible costs associated with *multiple* fitness-related traits. Within the next decade, it may be possible to limit crop-to-wild gene flow using crops with a suicide trait known as a "technology protection system" (sometimes referred to as the Terminator concept), but at present this controversial technology is still under development.

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Gene flow from oilseed rape to *Sinapis arvensis*: Variation at the population level

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ABSTRACT

Sinapis arvensis is a widespread weed species which is controlled by the use of herbicides. If oilseed rape crops containing transgenes for herbicide resistence are to be released, it is important to consider the consequences for weed control. Previous work on sexual compatibility between these two species has given variable results. The aim of this work is to investigate the likelihood of transgenes escaping from rape into *S. arvensis* populations. This paper reports the preliminary findings from crosses carried out between a transgenic rape line and *S. arvensis* populations collected from around the UK and France.

INTRODUCTION

As part of the risk assessment for genetically modified crops it is important to consider the species with which the crop will hybridise. Sexual compatibility between oilseed rape (*Brassica napus*) and related species has been reviewed (Scheffler & Dale, 1994). *Sinapis arvensis* was not considered to be sexually compatible because the majority of studies found that hybridisation between *S. arvensis* and *Brassica napus* is not possible without embryo rescue or ovule culture (Bing *et al.*, 1995; Bing *et al.*, 1996a; Bing *et al.*, 1996b; Brown & Brown, 1996; Kerlan *et al.*, 1992). However, Chevre *et al.* (1996) and Lefol *et al.* (1996) have found hybrids can be produced under open pollination conditions.

S. arvensis is a very widespread weed found in disturbed sites (Fogg, 1950). This means that it is frequently found growing in cultivated areas and was a serious weed before control by herbicides was achieved (Rich, 1991; Edwards, 1980). Herbicide tolerant oilseed rape may be one of the first GM crops to be released on a commercial scale.

For these reasons it is important to clarify the ability of *S. arvensis* to hybridise with oilseed rape. The differences found between the previous studies may reflect geographical variation in hybridisation ability so seed was collected from populations across the UK and from France where hybrids were previously found (Chevre *et al.*, 1996). This paper describes the preliminary work on the sexual compatibility of these populations with oilseed rape.

METHODS AND MATERIALS

Seed were collected from all populations of *S. arvensis* encountered when travelling around the UK in September/October 1997. Seed from French populations were supplied by A. Chevre (INRA, Rennes).

The oilseed rape plants used as pollen donors were Westar 10 transformed with a construct containing the BAR and NPTII genes conferring resistance to glufosinate ammonia and kanamycin, respectively. The plants were hemizygous for the transgene. Oilseed rape plants used as female parents were from the same line backcrossed to Westar 10, therefore, half of these plants contained the transgene.

Seed from 102 *S. arvensis* populations were grown and two plants from each population were used for the pollinations. For each plant, 30 buds were emasculated just before opening and pollinated with rape pollen. Each plant was also used to pollinate 20 emasculated rape buds. In addition, 16 plants were pollinated with other members of the same species to give a figure for the maximum number of seed obtainable under the experimental conditions. The pods were allowed to develop naturally in the glasshouse and seed harvested after 8 weeks for *S. arvensis* and 6 weeks for rape.

To date half of the seed have been sown. The resulting plants have been tested for presence of the transgenes by PCR using primers for BAR and NPTII. Leaf discs were cultured in the presence of the herbicide to test for glufosinate tolerance and to back up the PCR results.

RESULTS

Table 1 gives the location of the populations sampled. Dumfries and Galloway, Lancashire, Cheshire, Gwent, Somerset, Surrey, W. Glamorgan, Mid Glamorgan, S. Glamorgan, Devon and Cornwall were not visited. No *S. arvensis* populations were found in Gwynedd, Clwyd, Powys, Staffordshire, Derbyshire, Leicestershire, Hertfordshire, Essex, Avon or Hampshire

Table 1. The location of each population sampled and the number of seed produced in the interspecific crosses. The mean seed number is given for crosses involving two *S. arvensis* plants with 30 *S. arvensis* buds and 20 *B. napus* buds pollinated per plant. The number of the resulting seed which successfully germinated is given, out of the number of seed sown. Figures in brackets give the number of seeds germinating and immediately dying.

Population	Location	Mean no. seed/bud pollinated		No. of plants obtained /cross	
		Sinapis female	Sinapis male	Sinapis female	Sinapis male
1	Cambridgeshire	0	0	-	-
2	Cambridgeshire	0	0	-	-
3	Suffolk	0.033	0	2/2	-
4*	Norfolk	-	-	-	-
5	Norfolk	0	0	-	-
6	Cambridgeshire	0.033	0.100	-	-
7	Cambridgeshire	0	0	-	-
8	Cambridgeshire	0	0	-	-
9	Lincolnshire	0.017	0	1/1	-
10*	Lincolnshire	-	-	-	-
11	Lincolnshire	0	0	-	-
12	Lincolnshire	0	0	-	-

Table 1. continued

Population	Location	Mean no. seed/bud pollinated		No. of plants obtained /cross	
		Sinapis female	Sinapis male	Sinapis female	Sinapis male
13	Lincolnshire	0	0	-	-
14*	Lincolnshire	-	-	-	-
15	Lincolnshire	0	0	-	-
16	Lincolnshire	0	0.175	-	2(1)/7
17	Lincolnshire	0	0	-	-
18	Cambridgeshire	0	0	-	-
19	Kent	0	0	-	-
20	Kent	0	0	-	-
21	Kent	0.017	0	0/1	-
22	Kent	0	0	-	-
23	Kent	0	0	-	-
24	West Sussex	0	0	-	-
25	East Sussex	0	0	-	-
26	East Sussex	0	0	-	-
27	East Sussex	0.183	0	11/11	-
28	Kent	0.017	0.050	-	-
29	Norfolk	0	0	-	-
30	Norfolk	0	0	-	-
31	Norfolk	0.017	0	1/1	-
32	West Sussex	0	0	-	-
33	Norfolk	0.033	0	-	-
34	Suffolk	0	0.025	-	0/1
35	Suffolk	0	0	-	-
36	Norfolk	0	0.025	-	1/1
37	Norfolk	0	0	-	-
38	Norfolk	0	0	-	-
39	Norfolk	0	0	-	-
40	Cambridgeshire	0	0	-	-
41	Norfolk	0	0.025	-	0/1
42	Cambridgeshire	0	0	-	-
43	Northamptonshire	0.017	0.250	0/1	10/10
44	Warwickshire	0	0	-	-
45	Hereford and Worcester	0	0	-	-
46	Hereford and Worcester	0	0	-	-
47	Gloucestershire	0	0.100	-	4/4
48	Wiltshire	0	0	-	-
49	Dorset	0	0	-	-
50	Dorset	0.117	0	5(2)/7	-
51	Wiltshire	0	0.050	-	2/2
52	Oxfordshire	0	0	-	-
53	Oxfordshire	0	0	-	-
54	Oxfordshire	0	0	-	-
55	Buckinghamshire	0	0.050	-	-
56*	Berkshire	-	-	-	-
57	Wiltshire	0	0	-	-
58	Oxfordshire	0.167	0	-	-
59	Buckinghamshire	0	0	-	-
60	Buckinghamshire	0	0	-	-
61	Bedfordshire	0	0.025	-	1/1

Table 1. continued

Population	Location	Mean no. seed/bud pollinated		No. of plants obtained /cross	
		Sinapis female	Sinapis male	Sinapis female	Sinapis male
62	Bedfordshire	0	0	-	-
63	Lincolnshire	0	0.050	-	0/2
64	Nottinghamshire	0	0	-	-
65	South Yorkshire	0	0	-	-
66	Humberside	0.033	0.050	0/2	-
67	Humberside	0.183	0.025	-	-
68	Humberside	0	0.025	-	0/1
69	Humberside	0	0.025	-	0/1
70	Humberside	0	0	-	-
71	North Yorkshire	0	1.525	-	-
72	North Yorkshire	0	0.025	-	0/1
73	North Yorkshire	0	0	-	-
74	West Yorkshire	0	0	-	-
75	North Yorkshire	0	0	-	-
76	Durham	0	0	-	-
77	Northumberland	0.017	0.050	(1)/1	1/2
78	Northumberland	0.017	0	-	-
79	Northumberland	0	0	-	-
80	Borders	0	0	-	-
81	Borders	0	0.025	-	0/1
82	Lothian	0	0.175	-	-
83	Lothian	0	0	-	-
84	Fife	0	0	-	-
85	Tayside	0	0	-	-
86	Tayside	0	0.025	-	1/1
87	Grampian	0	0	-	-
88	Grampian	0	0	-	-
89	Grampian	0	0.050	-	2/2
90	Grampian	0.033	0	-	-
91	Grampian	0	0	-	-
92	Grampian	0	0	-	-
93	Grampian	0.067	0	2/5	-
94	Grampian	0.033	0	1(1)/2	-
95	Grampian	0	0	-	-
96	Highland	0	0.025	-	1/1
97	Highland	0	0	-	-
98	Highland	0	0	-	-
99	Highland	0	0.025	-	-
100	Highland	0	0	-	-
101	Highland	0	0	-	-
102	Strathclyde	0	0	-	-
103	Cumbria	0	0.475	-	1/3
104	Cumbria	0	0	-	-
105	Shropshire	0.100	0	-	-
106	Dyfed	0	0	-	-
Fr	France	0.083	0	-	-

* = populations producing no viable seed in the field in 1997.

Overall the number of seed produced per 100 buds was 1.1 for crosses where *S. arvensis* was the female and 3.4 for the reciprocal cross. The direction of the cross had no significant effect on the number of seed produced (Mann-Whitney, W=40654, p=0.157). There was no significant link between the ability of an individual *S. arvensis* plant to produce seed as the female parent and its success as the pollen donor (df=1, X^2=0.110, p>0.10). Control crosses within species produced 15,380 seed/100 buds for *S. arvensis* x *S. arvensis* and 33,360 seed/100 buds for oilseed rape x oilseed rape.

The results of the PCR and the BAR colour assay found that none of the potential hybrids produced from crosses with *S. arvensis* as the female parent contained the transgene. The rape parent was hemizygous for the transgene and so this result does not eliminate the possibility that these progeny are true hybrids. However, all of the potential hybrids had an identical morphology to that of the female parent.

DISCUSSION

S. arvensis plants were found across the country in areas where oilseed rape is grown and in areas where it is not. A very low number of seed were produced in the reciprocal crosses between *S. arvensis* and oilseed rape. These crosses involved *S. arvensis* plants from across the sampling area in regions with and without rape production. The hybrid status of these progeny has not yet been confirmed. These seed may have resulted from a number of processes other than hybridisation. They may be the result of contamination during the hand pollinations or matromorphy induced by intergeneric pollen as has been reported to occur in the Cruciferae (Banga, 1986). A range of molecular markers and root-tip chromosome counts are being used to investigate the hybrid status of these plants.

In previous studies where hybrids were obtained between these species, hybrids only resulted from crosses where oilseed rape was the female parent (Lefol *et al.*, 1996; Chevre *et al.*, 1996) and plants with a higher ploidy level are usually more successful as the female parent (Kerlan *et al.*, 1992). In this study more seed were produced when oilseed rape was the female parent but this was not significant.

The crosses discussed here form the preliminary survey of the *S. arvensis* populations and will not necessarily predict hybridisation in the field. If any of the progeny are confirmed as hybrids then more work will be carried out to determine whether hybridisation can occur without hand pollinations.

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