Gene flow from oilseed rape to weeds

A M Chèvre, F Eber, M Renard
Station d'Amélioration des Plantes, INRA, BP 29, 35650, Le Rheu, France

H Darmency
Unité de Malherbologie et Agronomie, INRA, BV 1540, 21034, Dijon, France

ABSTRACT

Gene flow between transgenic crops and their wild relatives is a key issue for the scientific and public acceptance of genetically modified varieties. The results reported in this paper concern the assessment of F1 interspecific hybrid production and the analysis of the following generations. The subjects of the studies were male sterile or male fertile herbicide tolerant oilseed rape cultivars (*Brassica napus*) and three weedy species largely spread in cultivated areas and presenting an overlapping flowering period. These were hoary mustard (*Hischfeldia incana*), wild mustard (*Sinapsis arvensis*) and wild radish (*Raphanus raphanistrum*). All the F1 interspecific hybrids were obtained except using wild mustard as the female but the rates of production depends on the weed and on the conditions of production. Experiments under normal agronomic conditions revealed a very low frequency of hybrids. The F1 hybrids are vigorous but their low female fertility reduces their fitness. From oilseed rape-wild radish hybrids, we studied the four following generations, under optimal conditions. We observed a reduction of the transgene transmission and of the chromosome number but an increase of the male and female fertility. However, none of the herbicide tolerant plants had the same chromosome number as the weed. Complementary experiments and strategies for reducing the gene flow are presented and discussed.

INTRODUCTION

One of the main concerns linked to the commercial release of herbicide resistant crops (HRC) is the likelihood of the spread of resistance genes to wild relatives of crops. This could create 'super-weeds' and make weed control more difficult. Since agronomists have seldom identified this kind of event in fields, the question is to estimate to what extent this phenomenon occurs naturally and how herbicide resistance can lead to troublesome consequences. It is important also to determine how to reduce the probability of occurrence of agronomic problems. In order to know if interspecific hybridisation and introgression are easy, different aspects have to be studied; (1) the existence of close relatives of the crop growing in sympatry, and the similarity between their phenology to provide overlap of flowering; (2) the nature of the mating system that allows pollen flow and allogamy; (3) the production of F1 interspecific hybrids and their survival; (4) the occurrence of fertile plants in the successive generations; (5) the possibility of chromosome recombination and spread of the gene in populations of the wild species.

There is little data available about gene flow from crops to related species. Evidence of past introgression is limited as it was not studied in detail. The question of gene flow is particularly relevant to oilseed rape (AACC, 2n=38) because this species is 20-40 % allogamous, produces a huge quantity of

pollen, 5 x 10^{12} pollen grain per ha, and outcrossing occurs through wind and insects. Simultaneously, numerous weedy species more or less related to oilseed rape are present within the cultivated areas with an overlapping flowering period, depending on the region and of the cultivars. For instance, early flowering cultivars had flowering time ending at the onset of flowering of wild mustard in Burgundy (France), that means there is no risk of cross-pollination, while late flowering cultivars overlaps for at least 25 % of their flowers with flowering of the weed (Lefol *et al.,* 1996b). An experimental approach is essential to provide the necessary insight into the topic. We show here an example of experimental research in which we try to bring new data and advice for good management at every step of the introgression between oilseed rape and some common *Brassicaceae* weeds.

METHODS AND MATERIALS

Different oilseed rape cultivars were used: either male sterile (Ogu-INRA system) or male fertile with or without a transgene (*bar* or *pat*) which confers the tolerance to the herbicide Basta® (glufosinate). The populations of weedy species, hoary mustard (AdAd, 2n=14), wild mustard (SarSar, 2n=18) and wild radish (RrRr, 2n=18), were locally collected in Rennes and Dijon regions (France).

Field experiments were performed during several years with different ratios of plants according to the optimal or agronomic conditions analysed in Rennes and Dijon. At each generation, the presence of the transgene was detected by herbicide spraying. The chromosome number was assessed by chromosome counting and/or flow cytometry (Eber *et al.,* 1997). The morphology of the plants was observed. The male fertility was assessed as the percentage of pollen stained by an aceto-carmine solution (at least 600 pollen grains/plant) and the female fertility (number of seeds per 100 flowers and per plant) was deduced from harvesting data.

RESULTS

F1 interspecific hybrid production

Between oilseed rape and different weeds

Under optimal conditions, using male sterile oilseed rape as female and the same ratio of crop and weed, the frequency of interspecific hybrids depends on the weed used as pollinator; it was assessed as 0.18, 1.9 and 23.8 interspecific hybrid seeds per 100 flowers using wild mustard, hoary mustard and wild radish as pollinators, respectively (Eber *et al.,* 1994; Chèvre *et al.,* 1996). Where the weed is the female and the oilseed rape the pollinator, a very low frequency of hybrid production was seen with hoary mustard (Lefol *et al.*, 1996a) and wild radish (Darmency *et al.*, 1998). Hybridisation on wild mustard was not detected (Lefol *et al.*, 1996b).

Between different oilseed rape cultivars and wild radish under optimal conditions

Using different male sterile oilseed rape cultivars containing the *bar* gene at the heterozygous stage and wild radish as pollinator, we observed that the F1 interspecific hybrid rates depends on the female parent; they ranged from 1.4 to 100.4 hybrid seeds/100 flowers and from 5.3 to 1213.3 hybrid seeds/plant (Baranger *et al.*, 1995).

Between an oilseed rape cultivar and wild radish under normal agronomic conditions

One hectare of Synergy cultivar glufosinate tolerant (80% of male sterile hybrid with two copies at the hemizygous stage + 20% a pure line with two copies at the homozygous stage) was sown. Wild radish plants were transplanted at different densities:

- within the field, either as isolated plants (1 plant/20m²) with 3 replicates or in groups (100 plants/50m²) with 3 replicates,
- in the border of the field, either as isolated plants (1 plant each 10 metres) or in groups of 40 plants on lines with three replicates,
- in the margin of the field, either as isolated plants (1 plant each 10 metres) or in groups 40 plants on lines with three replicates.

At least 20 metres between each treatment were maintained. Three fields were grown, two in Dijon during two years and one in Rennes. Whatever the location of the wild radish plants, a good overlapping of the flowering period was observed.

Wild radish was harvested plant by plant and the seedlings obtained from the seeds were herbicide treated either under greenhouse or field conditions. Among the total of 189,420 seedlings treated, only one hybrid at 2n=37 (ACRrRr) was detected that was glufosinate resistant. It was harvested on an isolated wild radish plant growing in the margin of the field. With 95% confidence limit, the expected frequency of hybrid plants among the total of observed plants, whatever the location of the wild radish mother plants, ranged from 10^{-7} to 3×10^{-5}.

In the Rennes trial, oilseed rape seed was harvested around the wild radish plants developed within the field or as clusters in the border or the margin of the field. According to our previous results, only the smallest seeds (diameter <1.6mm) were analysed. Two criteria of identification were used. In the greenhouse, the chromosome number assessed by flow cytometry and in the field, the duration of the flowering period, as interspecific hybrids flower longer than oilseed rape because of their female sterility, then the chromosome number of such plants was also checked by flow cytometry. Among seedlings characterised, a total of 23 hybrids were detected; 78% of them had 28 chromosomes (ACRr) but four amphidiploids (2n=56, AACCRrRr) and one 'BC1' like plant (2n=37, ACRrRr) were also obtained. Among the 73,847 plants obtained only from the smallest oilseed rape seeds harvested in the different locations around wild radish plants, the expected frequency of hybrid plants ranged from 2×10^{-5} to 5×10^{-4}, with 95% confidence limit. However, the smallest seeds represented only 7.3 to 12.3% of the total oilseed rape seeds harvested.

Analysis of the F1 interspecific hybrids and of the following generations

From the interspecific hybrids obtained between oilseed rape and hoary mustard

From oilseed rape-hoary mustard interspecific hybrid seeds, we observed that dormancy is intermediate between that of the crop and the weed (Chadoeuf *et al.*, 1998). The morphology of the F1 interspecific hybrid is very close to oilseed rape. This could explain why hybrids were seldom identified in the past. Hybrids are as good as, or even more, competitive than the weed (Lefol *et al.*, 1995), apart that seed output is very low, 0.2 seed per plant on average

(Lefol *et al.*, 1996a). This makes the fitness of hybrids very close to 10^{-6}. This should not be a troublesome problem if good weed management practices were effectively used.

From oilseed rape-wild radish hybrids

From the high number of glufosinate tolerant F1 interspecific hybrids (generation G1) obtained under optimal conditions, the four following generations were produced always with the same field design i.e. the same ratio of hybrids and of wild radish and the harvest was performed on the hybrids. From F1 interspecific hybrids (2n=28), we observed at the following G2 generation an increase of the chromosome number (Figure 1) and of the *bar* gene transmission (Figure 2) because of the efficiency of unreduced gametes (Chèvre *et al.*, 1997; 1998). However, the female fertility remained very low (Figure 2). During the following G3, G4 (Chèvre *et al.*, 1997) and G5 generations, the chromosome number decreased (Figure 1) and at the G5 generation, 91% of the herbicide tolerant hybrids had less than 23 chromosomes. Similarly, the percentage of glufosinate tolerant plants (Figure 2) decreased whereas the male and female (Figure 2) fertility increased. Some herbicide tolerant plants had a female fertility equivalent to wild radish but none of them had 18 chromosomes as wild radish. Their fitness was very low because they showed a chlorophyll deficiency, likely due to an incompatibility between the radish nucleus and the oilseed rape cytoplasm. The reciprocal phenomenon was already described by Pelletier *et al.* (1983).

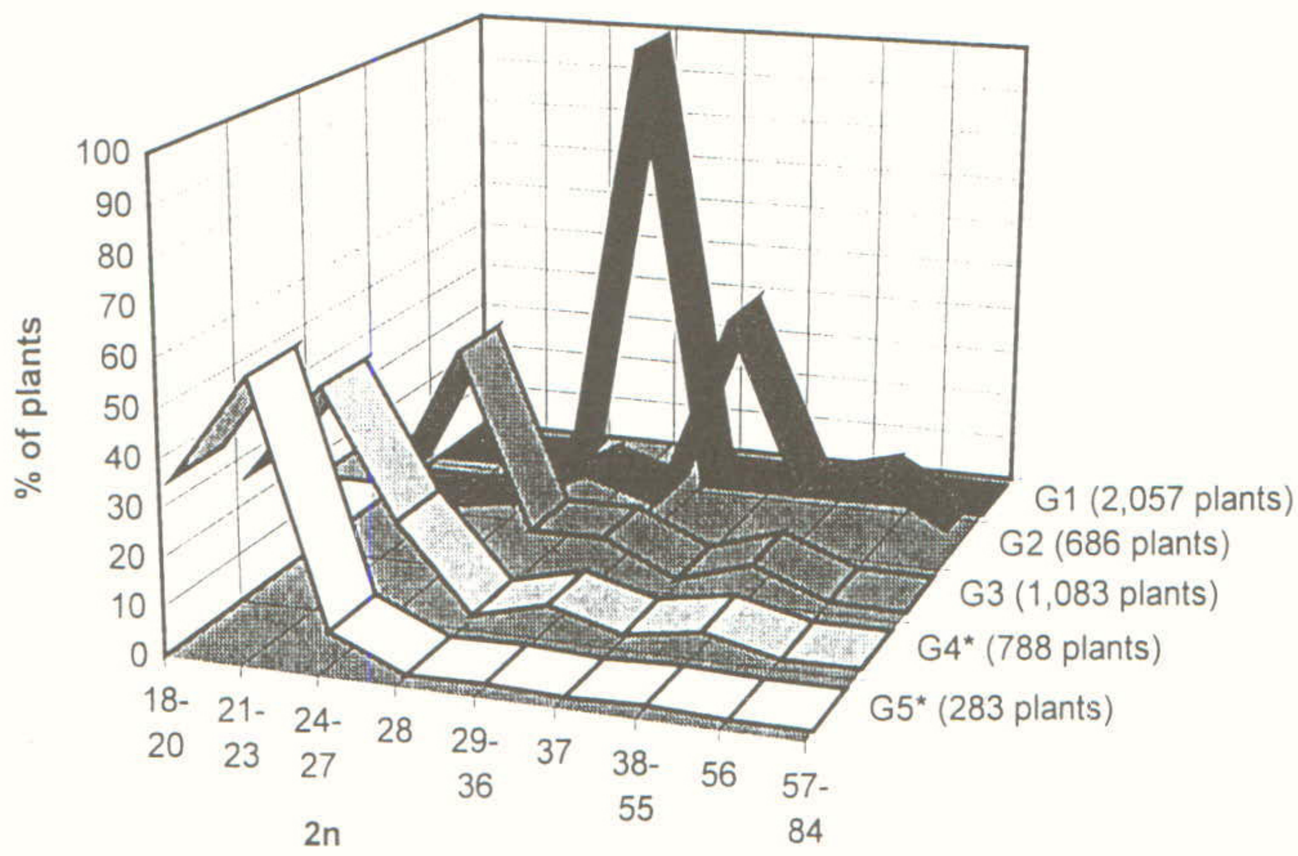

Figure 1. Evolution of the chromosome number along the different generations
* only the herbicide tolerant plants were analysed

DISCUSSION

From our experimental data, we showed that the production of interspecific hybrids between oilseed rape and weeds is possible but depends on; (1) the weed concerned and on the genotype of oilseed rape (Baranger *et al.*, 1995; Chèvre *et al.*, 1996); (2) the female parent since usually more seeds are produced using oilseed rape as female (Kerlan *et al,* 1992), (3) the variability in the weeds (Lefol *et al.*,1996a; Darmency *et al.*,1998); (4) the spatial

relationship between the crop and the weed species (Lefol *et al.*,1996a; Darmency *et al.*,1998). These observations are in agreement with the results reported by other authors (Jorgensen & Andersen 1994; Bing *et al.*, 1996; Jorgensen *et al.*, 1998). However, usually, the hybrid production on the weeds and under normal agronomic conditions remains low.

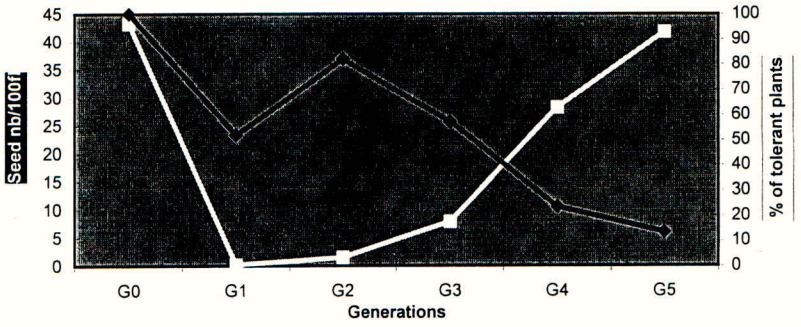

Figure 2. Evolution of the female fertility (white line) assessed by the number of seeds/100 flowers and of the percentage of herbicide tolerant plants (black line) along the different generations.

Triploid F1 interspecific hybrids are vigorous but have a low fertility. However, the female fertility of amphidiploid plants (AACCRrRr, 2n=56) which were obtained under normal agronomic conditions and which had an oilseed rape cytoplasm and a male fertility equivalent to oilseed rape (data not shown) remains to be checked.

The analysis of the following generations from oilseed rape-wild radish F1 interspecific hybrids revealed that, at the fifth generation, none of the herbicide tolerant plants had the same chromosome number as the weed. So, the transgene was not established in the genome of the weed (Chèvre *et al.*, 1997). However, only one insertion event was used to produce this material and our analysis of different specific oilseed rape loci indicated that their transmission rates are different according to the locus (Chèvre *et al.*, 1998). Further analysis is needed to investigate the effect of the initial location of the transgene on gene introgression into the genome of a close related species.

Different models are under study to integrate the different genetic parameters, the spatial and temporal effects using data from small-scale field trials. However, the main difficulty remains the validation of the data from large-scale experiments to get accurate models that could be used for prediction.

In addition, different strategies are under study for reducing the risks of gene flow which include (1) the identification of a potential 'safe insertion site' for the transferred gene, (2) modifications which could reduce the dispersal of pollen (e.g. self-fertilising cultivars) and seeds (e.g. reducing seed loss at harvesting and dormancy), (3) the adaptation of agronomic practices (e.g. management strategies for herbicide tolerant volunteers).

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Gene flow from transgenic canola to wild radish - a model system to determine the risks

M A Rieger, C Preston
The Co-operative Research Centre for Weed Management Systems, University of Adelaide PMB 1 Glen Osmond, 5064 Australia

T Potter
South Australian Research and Development Institute, Struan Research Station, Naracoorte 5271, Australia

S B Powles
WA Herbicide Resistance Initiative, University of Western Australia, Nedlands, Perth 6907, Australia

ABSTRACT

Under Australian field conditions, gene flow between canola (*Brassica napus*) and a related weed species, wild radish (*Raphanus raphanistrum*) was examined. A model system without transgenic material but utilising herbicide resistance was employed. In the first year of this study a total of five putative hybrids have been discovered. The morphology, pollen fertility and seed set of these individuals is discussed.

INTRODUCTION

Crops such as wheat, barley and grain legumes dominate the Southern Australian broad-acre cropping system. Canola (*Brassica napus*) is a more recent introduction into this system and plantings have steadily increased in area over the last five years from 107,000 ha in 1993 to over 1 million ha in 1998. In some areas, canola is replacing grain legumes as a break crop in cereal rotations. Canola has been introduced into a farming system with a background of extensive herbicide resistant weed populations. The most prominent among these weeds is annual rye-grass (*Lolium rigidium*). This highly adaptable weed now has populations resistant to most herbicides, including glyphosate, used in Australian cropping systems. Another major weed is wild radish (*Raphanus raphanistrum*). Populations of wild radish resistant to acetolactate synthase (ALS) herbicides have recently been documented. These two weed threats have led to the widespread adoption of triazine tolerant canola varieties, which account for nearly 50% of all canola plantings. These lines contain a triazine-resistance gene from a weedy relative (*Brassica rapa*) that was crossed into canola.

The high adoption rate of triazine-tolerant canola varieties over the past three years demonstrates that growers are receptive to this technology despite the penalties of lower yields and oil contents. It is into this environment that other herbicide-resistant canola varieties will be introduced into Australia over the next few years. Imidazolinone tolerant canola should be introduced in 2000 or 2001, closely followed by the transgenic glufosinate resistant and glyphosate resistant varieties.

A major concern with canola is its ability to out-cross to weedy relatives that infest canola crops. For this reason, there has been intense research interest investigating the possibility of transgenic herbicide-resistant genes flowing from canola to weedy relatives (Jorgensen & Andersen, 1994; Brown & Brown, 1996; Mikkelsen *et al.*, 1996a & 1996b). By and large, much of this research has failed to mimic normal cropping situations and has often used male-sterile canola varieties (Eber *et al.*, 1994; Baranger *et al.*, 1995; Darmency *et al.*, 1995; Lefol *et al.*, 1997). The use of male-sterile varieties increases that probability of out-crossing by the elimination of competition from canola pollen. With male-fertile canola it is more difficult to determine hybrid production; however, this mimics the field situation more accurately. Further there is a need to incorporate weeds at realistic field densities to ensure the rates of hybridisation are not overestimated. This paper will focus on the likelihood of hybridisation from canola to wild radish with experimental "in-crop" field trials and using a male-fertile variety of canola.

METHODS AND MATERIALS

Field trials located at Naracoorte (South Australia) commenced in autumn 1997 and will continue over two consecutive seasons. No transgenic material was used in the field trials due to regulatory constraints. In the absence of transgenic material, an alternative marker system, resistance to sulfonlyurea and imidazolinone herbicides, was utilised. Gene flow was also examined in both directions, from canola to wild radish and from wild radish to canola.

Direction a: gene flow from canola to wild radish

A field was planted to traditionally bred imidazoline-resistant canola (cultivar 45A71) and subsequently inter-planted with susceptible wild radish at one and four plants/m^2. There were a total of eight 10m x 10m plots and wild radish seeds were hand collected from plants prior to crop harvest.

Direction b: gene flow from wild radish to canola

A field was planted to ALS-herbicide susceptible canola (cultivar Karoo) and inter-planted with ALS-resistant wild radish plants at one and four plants/m^2. There were fifteen 10m x 10m plots. After flowering was completed the resistant wild radish plants were removed. The canola seed was harvested mechanically at the regular harvest time.

Screening of the wild radish seed - direction a

The wild radish seeds collected from all plots were kept separate for each individual plant. Wild radish seeds are known to be highly dormant and were pre-treated to break dormancy. This pre-treatment involved a 10 minute soak in 6% sodium hypochlorite with an additional period soaking in water. Pre-treated seeds were then germinated on 1.6% water agar with 5 x 10^{-5} M ALS-inhibiting herbicide (imazethapyr) incorporated. Seeds were allowed to germinate at 25°C/15°C in an incubator for a period of three weeks. Assessment of root growth occurred after this period. The roots of susceptible wild radish are severely stunted if seed is germinated in the presence of this concentration of herbicide. Plant with roots >10 mm long were classified as resistant and transplanted to soil (screen one). Once the survivors

of screen one had reached the two/three leaf stage they were treated with 48 g/ha imazethapyr *via* a belt driven laboratory spray unit. Survival was assessed at approximately one week after spray application (screen two).

Screening of the canola seed - direction b

Canola seed was machine harvested and separated for each of the 15 plots. In the following season the canola seed was planted out in plots and allowed to germinate. The number of plants per plot was determined based on the area of each plot and counting the number of plants present in twenty 10 x 10 cm quadrats. Once these plants had reached the two/three leaf stage the area was sprayed with two applications of 7.5 g/ha chlorsulfuron in alternate directions to ensure even coverage. A week later an additional application of 15 g/ha of chlorsulfuron was made. These plots were then left for a period of three weeks to allow the herbicide-susceptible seedlings to die (screen one). Surviving plants in these plots were transplanted to pots and two weeks after transplantation were again treated with 15 g/ha of chlorsulfuron (screen two).

Hybrid confirmation

A suite of verification methods will be used for hybrid confirmation including observations of morphology, flowering and cross fertility along with the use of molecular techniques. Molecular techniques will use the presence of a mutation endowing herbicide resistance in the ALS gene as the main identification tool. If the mutations within ALS are identical to those of the putative resistant parent species, other parts of the ALS gene will be examined to determine whether it came from the resistant parent.

RESULTS

Direction a

A total of 38,635 wild radish seeds were processed through screen one. Of these 19,036 (49.3%) seeds germinated and 5,631 (14.6%) were transplanted to soil. A total of 6 plants survived the transplantation step and after screen two, 2 individuals (A1, A2) remained. These two putative hybrids did not differ significantly in morphology from canola. Pollen viability was examined using the fluoresceine diacetate method. Three separate flowers were examined per plant, and at least 500 pollen grains counted per flower (Table 1). Both hybrids produced pods when crossed with wild radish and canola (Table 2).

Direction b

An estimated number of 51 million seeds were processed through screen one. Two thousand five hundred and seventy plants survived this first screen and were subsequently transplanted into pots. Most of the plants surviving screen one were visually identical with canola, but three plants (B1, B2, B3) were morphologically different. These plants had many characteristics in common with wild radish including differences in leaf shape, hairiness of leaves, flower structure, and pod constriction. These putative hybrids clearly demonstrated morphology that was intermediate between wild radish and canola. Only these three plants

survived screen two. The field where screen one had been performed was subsequently sown to wheat. After two months seed from further 86 plants were collected from this field and will be screened for potential herbicide-resistance. The fluoresceine diacetate method was used to examine pollen viability of these potential hybrid plants (Table 1). All putative hybrids produced pods when crossed with both canola and wild radish (Table 2).

Table 1. The percent pollen viability of putative hybrids based on three replicates.

	% viability			% overall
A1	49	81	78	70
A2	51.5	27	52	43.5
B1	39	60	63	63
B2	53	66	69	63
B3	30	80	83	64
Canola	59	56	59	58
Wild radish	73.5	76	63	71

Table 2. The number of pods produced by putative hybrids after crossing with canola and wild radish pollen. Number of selfed pods produced also shown.

	Self	x canola	x wild radish
A1	27	44	16
A2	69	12	62
B1	88	89	55
B2	41	12	42
B3	24	9	5

DISCUSSION

The potential hybrids produced by the first year of this field trial under Australian conditions indeed suggest that hybrid production between canola and wild radish is possible. The implications of these results are two-fold. Firstly based on research conducted into the evolution of herbicide resistance, the mutation rate one would expect for ALS-resistance is 1 x 10^{-6} (Jasieniuk *et. al.*, 1996). Numbers from these initial trials suggest that the rates of

hybridisation *via* gene flow are lower than selection for herbicide resistance through a mutation. This would indicate that weed species would be more likely to develop herbicide resistance *via* a mutation before hybridisation. Secondly if the plants produced by these field trials are in fact hybrids, two facets of their biology needs to be considered. All the potential hybrids produced by this study are reasonably fertile and all produced selfed seeds. The implication of this could be that once hybrids have been established through gene flow, spread of these individuals would be rapid. Instead of only one mechanism of weeds developing herbicide resistance, mutations through selection pressure, there may now be a slower but no less important mechanism, hybridisation through gene flow.

The methodology used during this trial has proved quite successful. Two years of these trials have now been conducted and only minor modifications were necessary in the second year. These modifications included increasing the number of replicates per treatment, modifying the method of collection of the wild radish seed and the addition of fungicide to avoid unnecessary seedling loss. Due to the success of this method additional trials have been planned. These future trials are to examine hybridisation between canola and several other important weed species in South Australia, namely *Brassica tournefortii* and *Diplotaxis murialis*.

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