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Hybridisation between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic field conditions

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Abstract The frequency of hybridisation between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic conditions was assessed in field experiments, where *R. raphanistrum* were randomly planted at two different densities into large plots of *B. napus*. An acetolacate synthase (ALS)-inhibiting herbicide-resistant trait was used to detect potential hybrid individuals. No hybrids were detected amongst 25,000 seedlings grown from seed collected from *R. raphanistrum* plants. Two hybrids were obtained from more than 52-million *B. napus* seedlings. Both hybrids were characterised as amphidiploids (AACCRrRr, 2n = 56) and were fertile. The frequency of hybridisation into *B. napus* in this experiment using male-fertile *B. napus* was 4×10^{-8}.

Keywords *Brassica napus* · *Raphanus raphanistrum* · Hybridisation rate · Agronomic conditions

Introduction

Transgenic crops are now grown in various parts of the world, with the majority occurring in North and South America, Canada and Australia (James 1998). For transgenic crops, as with any new technology, various risks have been raised (Colwell et al. 1985; Crawley 1993; Ellstrand 1993; Rissler and Mellon 1996). The potential for hybridisation of transgenic crops with weedy species and the subsequent introgression of transgenes has received considerable attention (Kapteijns 1993; Rogers and Parkes 1995; Whitton et al. 1997). Early research on agricultural hybridisation focused on the potential for weedy traits to contaminate crops (Haskell 1943; Bateman 1947; Nieuworf 1963; Barrnett 1983). With the development of transgenic crops, this emphasis has shifted to examine the possible escape of transgenes from the crop into weedy species. (Langevin et al. 1990; Klinger et al. 1991; Arias and Rieseberg 1994). The majority of crop-weed hybridisation studies have examined interspecific hybridisation between closely related species, while limited information is available on intergeneric hybridisation and the barriers that prevent hybrid production.

Both interspecific and intergeneric hybridisation between *Brassica napus* L. (AACC, 2n = 38) and several wild relatives are known (Heyn 1977; Prakash and Hinata 1980). *B. napus* is known to be predominantly self-fertile but with the potential for 30% outcrossing (Williams et al. 1986; Rakow and Woods 1987). Viable hybrids can be produced when *B. napus* is crossed with either *Brassica juncea* (Roy 1984; Sacristan and Gerdemann 1986; Frello et al. 1995) or *B. rapa* (Jorgensen and Andersen 1994; Bing et al. 1996; Brown and Brown 1996; Mikkelsen et al. 1996) in either direction. Partially fertile hybrids from wider crosses such as *Raphanus raphanistrum* L. (RrRr, 2n = 18) (Kerlan et al. 1992; Chevre et al. 1994; Baranger et al. 1995) and *Sinapis arvensis* L. (SarSar, 2n = 18) (Kerlan et al. 1992; Bing et al. 1995; Lefol et al. 1996) have also been produced. For hybrids with *R. raphanistrum* a combination of hand-pollination and embryo rescue is normally required when using *B. napus* as the female parent (Kerlan et al. 1992; Chevre et al. 1996). Lower numbers of hybrids are obtained when embryo rescue is not used (Darmency et al. 1995; Lefol et al. 1997). Field trials

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have shown surprisingly large amounts of hybridisation but male-sterile *B. napus* has been used (Eber et al. 1994; Chevre et al. 1996). When using *B. napus* as the male parent, hybrid production is higher both with (Kerlan et al. 1992; Chevre et al. 1996) and without (Lefol et al. 1997) embryo rescue. Studies of open pollinations using *B. napus* as the male parent are very limited (Darmency et al. 1995; Chevre et al. 2000). Crossing into *R. raphanistrum* Darmency et al. (1995) obtained two hybrids from a sample of 956 seeds, using the variety Brutor. Chevre et al. (2000) observed a single hybrid individual after assessing 63,484 wild radish seeds. We report experiments conducted under commercial agronomic conditions investigating the frequency of hybridisation between male-fertile *B. napus* and *R. raphanistrum* in both directions, and discuss how these results will affect the management of transgenic *B. napus* varieties.

Materials and methods

Field trials were located near Naracoorte, South Australia (37°06′ 140°47′), during two growing seasons 1997–1998 and 1998–1999. Transgenic crop plants were not used due to regulatory constraints. Instead, non-transgenic plants possessing two homozygous dominant herbicide resistance genes, with a pair of alleles per genome, endowing resistance to certain acetolacate synthase (ALS)-inhibiting herbicides were employed. To examine the potential gene flow from crop to weed (direction A), a field of ALS herbicide-resistant canola (cultivar 45A71) containing the two homozygous resistance genes was subdivided into eight (97–98) or 12 plots (98–99), each 10 m × 10 m. Into these plots 4-week-old glasshouse-grown ALS herbicide-susceptible *R. raphanistrum* were inter-planted at two densities, 1 and 4 plants/m^2. To examine gene flow from the weedy species to the crop (direction B), a field of ALS herbicide-susceptible canola (variety Karoo) was planted and subdivided into 15 10 m × 10 m plots. These plots were inter-planted with ALS herbicide-resistant *R. raphanistrum* plants at the same densities as listed above. The *R. raphanistrum* used was a mixture from ten different populations sourced from Western Australia. In both canola fields, several control plots were maintained without *R. raphanistrum*. The *B. napus* and *R. raphanistrum* flowered synchronously, in both years. Once flowering was completed, seeds from the ALS herbicide-susceptible *R. raphanistrum* (direction A) were collected separately from each individual and the *B. napus* seed (direction B) was harvested mechanically following hand-removal of *R. raphanistrum*. Each of the different seed types was screened separately for resistance to ALS-inhibiting herbicides. A two-tiered screening method was employed to eliminate false positives and increase the rigour of the technique.

Screening of *R. raphanistrum* seed

ALS herbicides are known to inhibit the root growth of sensitive plants (Saari et al. 1994) and we used this to establish an agar-based assay. To determine the most-appropriate herbicide concentration that would distinguish between susceptible and resistant individuals, seeds were germinated on 0.6% (w/v) water agar at a range of concentrations (1–200 µM) of imazethapyr. Seeds were mechanically de-hulled and placed on the agar surface and allowed to germinate over 7 days in an incubator set at 25°C/15°C, with a photoperiod of 12 h. Both ALS herbicide-resistant and -susceptible seeds of *B. napus* and *R. raphanistrum* were tested. Initial germination was unaffected by the incorporated herbicide but subsequent root growth was inhibited in ALS herbicide-susceptible plants. A concentration of 5 µM of imazethapyr was ideal to distinguish between the resistant and susceptible individuals. The roots of susceptible individuals were severely stunted or sometimes non-existent. Resistance was determined using the length of root growth after a 3-week germination period, along with the general health of each seedling. Plants with roots >10-mm long were classified as resistant and transplanted to soil (screen one). Once the survivors of this first screen had reached the two/three-leaf stage, they were treated with 48 g/ha of imazethapyr using a custom-built moving-belt sprayer. This unit has an output of 124.5 l/ha when sprayed at 1 m/s and a pressure of 250 kpa. The survival of individuals was assessed 3 weeks after spray application (screen two).

Screening of the *B. napus* seed

The mechanically harvested *B. napus* seed was sown at 40–50 kg/ha^{-1} in the autumn following harvest, with each plot planted separately. The number of plants per plot was determined based on the area of each plot and counting the number of plants present in 20 randomly selected 10 × 10-cm quadrats. Once these plants had reached the two/three-leaf stage, the area was sprayed with two applications of the ALS herbicide chlorsulfuron (7.5 g/ha) in alternate directions to ensure even coverage. A week later an additional application of chlorsulfuron (15 g/ha) was made. These plots were left for a period of between 3 and 8 weeks depending on weather conditions (screen one). Surviving plants in these plots were transplanted to pots. After a 2-week recovery period, these plants were again treated with 15 g/ha of chlorsulfuron (screen two) using the laboratory sprayer. This rigorous screening (three separate herbicide treatments) ensured that only ALS herbicide-resistant individuals were selected.

Determination of hybrid status

The hybrid status of all surviving individuals from both directions was determined by examining the chromosome number, pollen viability and molecular markers, and an examination of various morphological features.

Cytogenetic studies

Unopened flower buds were collected early in the morning and fixed in 3:1 ethanol: acetic acid for a minimum of 24 h. For long-term storage the material was transferred to 70% ethanol and placed at 4°C. All six anthers were dissected from the surrounding floral material and the pollen mother cells were removed onto a clean slide. A single drop of 1% aceto-orcein was added and the mixture was allowed to stain for 1–2 min. A cover slip was applied and the slide examined using a Leitz binocular microscope and the chromosomes counted. At least 20 cells per individual were counted to verify the chromosome number. Pollen viability was examined using fluorescein diacetate (Heslop-Harrison and Heslop-Harrison 1970). Three separate flowers were examined per plant, and at least 500 pollen grains counted per flower.

RFLP analysis

DNA was extracted, using a scaled-down method of Kim et al. (1990), from the putative hybrids, two varieties of *B. napus* and two randomly selected *R. raphanistrum* plants. Contaminating RNA was removed from all samples using a 1:100 dilution of RNAase A (10 mg/ml). Restriction-enzyme digests were carried out with *Hae*III (Promega) following the manufacturer's instructions. Electrophoresis was carried out using a 1% agarose gel for 40 h at a constant 34 V. Southern blots on Hybond-N were hybridised to the M13 bacteriophage probe using the method outlined in Weihe et al. (1990).

Morphology analysis

During the growth period, the following characteristics were observed for parents and potential hybrids: whole-plant growth habit, glaucous/scabrous leaf characteristics, leaf shape and texture. Once flowering commenced, observations were made on inflorescence type, sepals, and floral veination. Pod and seed characters were observed.

Results

Potential hybridisation from *B. napus* to *R. raphanistrum* (direction A)

Over the 2-year period approximately 61,000 *R. raphanistrum* seeds were collected and individually evaluated (Table 1). In the 1998 experiment, 38,635 seeds were processed through the first screen. Of these 19,036 (49.3%) seeds successfully germinated and 5,631 (14.6%) displayed root growth in the presence of ALS herbicide and were taken to the second screen. Of those 5,631 plants, only two individuals survived the ALS herbicide treatment. In the second year of the trials (1999) 24,093 seeds were processed, 10,775 (44.7%) germinated and 1,439 (5.9%) survived the first herbicide screen and were transplanted. Only two plants survived transplantation and the second herbicide screen. Thus from a total of 62,728 seeds collected from ALS herbicide-susceptible *R. raphanistrum* plants growing with an ALS herbicide-resistant canola crop, only four individuals survived exhaustive ALS herbicide screening. These four individuals had chromosome counts (2n = 38) and morphological features identical to canola variety 45A71, with RFLP profiles confirming this (Fig. 1). These plants had an erect growth habit, with a single main stem. The leaf texture and flower shape/colour were identical to *B. napus* (Table 2); in addition, the seeds were dark in colour, lacking the reddish colour of *R. raphanistrum* seeds. These plants also lacked the characteristic pod constrictions associated with *R. raphanistrum*. During the harvesting of *R. raphanistrum* seed, canola plants were still present at the trial sites. Canola pods are prone to shattering and, as *R. raphanistrum* seeds were being collected, canola may have contaminated the *R. raphanistrum* seed. This is the probable cause of identifying canola variety 45A71 among the *R. raphanistrum* seed. It was therefore concluded that there was no evidence of hybridisation from *B. napus* to *R. raphanistrum.*

Potential hybridisation from *R. raphanistrum* to *B. napus* (direction B)

A total of approximately 66-million canola seeds germinated from the seed sown in the field over 2 years. Of these, 13.5-million seeds were generated from the control treatments and were not considered for subsequent analysis (Table 1). In the 1998 experiments, approximately 32-million seeds germinated from eight plots and 2,570 plants were collected after repeated ALS herbicide treatment. In 1999, over 12 plots, 20-million seeds germinated, with 30 plants collected after ALS herbicide treatment. From approximately 52-million seeds collect-

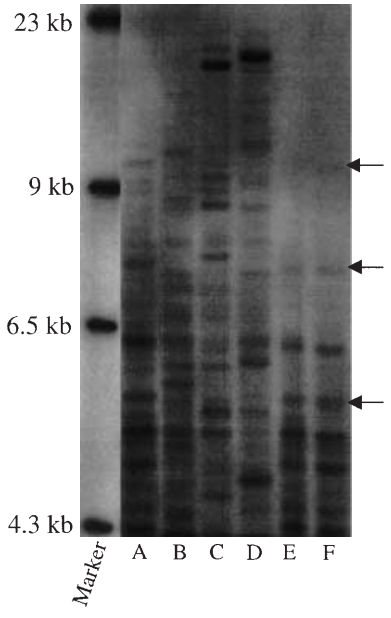

Fig. 1 Restriction length polymorphism of various putative hybrids probed with M13. Marker: *Hind*III ladder; *lane A* individual from direction a, determined to be the same as variety 45A71; *lane B* H1 from direction b; *lane C R. raphanistrum* from direction b; *lane D H2* from direction b; *lane E B. napus* variety 45A71; *lane F B. napus* variety oscar. *Arrows* indicate *Brassica*-specific bands

Table 1 Numbers of seedlings which germinate from *B. napus* and *R. raphanistrum* plants and the number of hybrids detected

Densities of *R. raphanistrum* (plants m^{-2})	No. of seedlings (97–98)	No. of hybrids	No. of seedlings (98–99)	No. of hybrids
Direction A (*B. napus*)				
0	13,468,160	0	0	0
1	13,582,070	0	11,116,300	0
4	18,536,400	2	8,903,413	0
Direction B (*R.raphanistrum*)				
0	4,710	0	0	0
1	2,666	0	4,818	0
4	11,660	0	5,957	0

Table 2 Morphological comparison between *R. raphanistrum*, *B. napus* and hybrids H1 and H2

Characteristic	*R. raphanistrum*	H1	H2	*B. napus*
Growth habit	Slender/prostrate	Slender/erect	Slender/prostrate	Robust/erect
Plant type	Multi-stemed	Multi	Multi	One main stem
Lower stem leaf shape	Lyrate-pinnatifid; lobes 5–7	Lyrate-pinnatifid; lobes 2–3	Lyrate-pinnatifid; lobes 2–3	Lyrate-pinnatifid; lobes 1–3
Upper stem leaf shape	Reduced, with stems still present	Entire-lanceolate	Entire-lanceolate	Oblong-lanceolate, stem Clasping
Leaf texture	Scabrous	Smooth, glabrous	Smooth, glabrous	glabrous, leathery, smooth
Inflorescence	Even	Paired	Even	Paired
Veination colouring	Dark	Light	Light	Light
Petal shape	Long clawed	Clawed	Long clawed	Clawed
Petal colour	White, pale yellow, purple	Yellow	Yellow/purple	Bright yellow

Table 3 Pollen viability of H1 and H2 compared with *B. napus* and *R. raphanistrum*

Individual	% Variability			
	Replicate 1	Replicate 2	Replicate 3	Average
H1	39	60	63	63
H2	30	80	83	64
B. napus	59	56	59	58
R. raphanistrum	73.5	76	63	71

ed from ALS herbicide-susceptible canola plants grown in the presence of ALS herbicide-resistant *R. raphanistrum*, only three individuals survived extensive herbicide screening. These three individuals were classified as putative *Raphanus-Brassica* hybrids. Two of the individuals proved to be true hybrids, with chromosome counts of 2n = 56 and numerous morphological features which resembled *B. napus*, including lack of hairs and leaf shape, but also with some intermediate characteristics resembling *R. raphanistrum* (Table 2). These features included a multi-stemmed and prostrate growth habit, smooth leaf texture, floral and seed colourations along with an intermediate pod shape. The third individual had a chromosome number of 2n = 18 and the morphological features of *R. raphanistrum*. The RFLP profiles for these two individuals (Fig. 1) are distinctly different from canola varieties 45A71 and Oscar. Pollen viability (Table 3) showed variation between both flowers and individuals, but both hybrids had viable pollen and were able to set seed when backcrossed to *R. raphanistrum* but not *B. napus*. Both hybrid individuals were also able to produce seed via selfing.

Discussion

The two hybrids between *R. raphanistrum* and *B. napus* were detected from a total sample size of approximately 52-million. The two hybrids detected translate to a hybridisation frequency of 4×10^{-8}. This compares with a frequency range of 2×10^{-5} to 5×10^{-4} recently reported by Chevre et al. (2000). The lower frequency reported by this study is due to the use of fully male-fertile *B. napus* and represents the lower limit of the hybridisation potential expected under commercial agronomic conditions. Contrary to reports by Baranger et al. (1995) and Chevre et al. (2000), no visible seed dimorphism was detected.

That hybridisation is more likely to occur between *B. napus* and *R. raphanistrum* when *B. napus* is the maternal parent is supported by the literature (see reviews by Scheffler and Dale 1994; Rieger et al. 1999). Kerlan et al. (1992) showed that 34% of *R. raphanistrum* pollen adhering to the stigma of *B. napus* germinated, but no pollen tubes penetrated the pistil. This pollen germination rate is similar to that of the *B. napus* self-compatible close relative *Brassica oleracea*. When *B. napus* pollen is applied to the stigma of *R. raphanistrum* only 12% of the pollen germinates. Contrastingly, *B. napus* pollen will readily germinate on the stigmatic surface of *B. oleracea* (Kerlan et al. 1992). This result indicates a directional preference in pollen germination between *B. napus* and *R. raphanistrum* at the level of the stigma. With regard to hybrid formation, both Chevre et al. (1996) and Lefol et al. (1997) found no statistical difference between reciprocal crosses, though more hybrid individuals were produced with *B. napus* as the female.

There are several possible mechanisms for this directional preference, which will ensure most of the hybridisation between these two species will occur into the crop species. Two hypotheses, unilateral pollen-pistil incompatibility (UI) and endosperm balance number (EBN), provide some explanation for the directional preference of the hybridisation potential between *R. raphanistrum* and *B. napus*. Previously, directional preference was either not observed, due to low samples sizes (Chevre et al. 1996), or explained by the mismatching ploidy level of the species involved (Kerlan et al. 1992). We argue that the mechanism of UI (Lewis and Crowe

1958) may also be functioning in this system. This phenomenon, described as early as 1955 (Harrison and Darby 1955), is explained as the pollen of one species rejecting the pistil of another, with the reciprocal direction being compatible (De Nettancourt 1977). Most commonly, UI occurs with a self-incompatible (SI) species as the pistillate parent and the self-compatible (SC) species donating the pollen. Such a mechanism may be responsible for the reproductive isolation between *B. napus* and the self-incompatible species *R. raphanistrum* (Kercher and Conner 1996).

The successful formation of an embryo is not only dependent on fertilisation but also on normal development of the endosperm. The endosperms of intraspecific and interploidy crosses often develop abnormally when there is a deviation from a 2:1 maternal to paternal ratio (Masuelli and Camadro 1997; Vancreij et al. 1997; Scott et al. 1998). Interspecific crosses are more complex and have led to the development of the EBN hypothesis (Johnston et al. 1980; Ehlenfeldt and Ortiz 1995; Carputo et al. 1999). This hypothesis extends the 2:1 ratio; the genome of each species is assigned an EBN which may be different for species of the same ploidy. It is the EBN which determines the effective ploidy of the endosperm and which must conform to the 2:1 maternal to paternal ratio. Johnston et al. (1980) tested the EBN hypothesis on various species of potato, and clarified previous results obtained in several other genera. Observations during the present study indicate that endosperm formation with seeds collected from *R. raphanistrum* was poor, with many of the seeds shrivelled and failing to germinate (data not shown).

Hybridisation is affected by many factors and the genetic background of the species involved is clearly one of these (Baranger et al. 1995). The *B. napus* cultivar used can affect the production of hybrids and Baranger et al. (1995) found that this explained a significant amount of the variation in hybrid production. For the present study, a single but different *B. napus* cultivar was used in each direction. A greater range of genetic diversity was available for *R. raphanistrum*, these plants being a mixture of ten populations sourced from Western Australia. The genetic diversity of *R. raphanistrum* in Australia is thought to be large, mainly based on morphological observations. An example is the variety of floral colours, with bronze, yellow, white, pink and mauve flowers observed (Cheam 1996; personal communication R. Cousens). The large range of genetic variability used during this study should not be present in all populations of *R. raphanistrum* located near *B. napus*. Based on the two hypotheses presented above, and the many factors which effect hybridisation, it is expected that most of the gene flow between these two species will be in one direction, from the weed into the crop. Therefore, the probability of weeds being pollinated by *B. napus* is likely to be less than 4×10^{-8}.

Since hybridisation is more likely into *B. napus*, hybrid individuals are only expected to occur in the crop. Most of this seed will be harvested and therefore only a small proportion of the original seed will remain. Hybrid individuals are expected to occur intermingled with *B. napus* volunteers in the following year. A number of alternative herbicides are available to control both types of volunteers. Due to the high fertility of the hybrids produced, these plants may become a bridge for gene escape into *R. raphanistrum* or become weedy themselves. Limited genetic variation, and hence crossing-compatibility in weed populations, may mitigate these gene escapes. If farmers concentrate on limiting volunteers for several years after growing herbicide-resistant *B. napus* varieties this will reduce the likelihood of gene escape via hybridisation. In conclusion, this study of hybridisation between *B. napus* and *R. raphanistrum* has revealed three important points. Firstly, hybridisation with male-fertile *B. napus* under field conditions occurs at a frequency of 4×10^{-8}, crosses are more likely if *B. napus* is the female parent and fertile amphidiploids can be produced under field conditions.

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