Introgression of genes from rape to wild turnip

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By
Toni E Jenkins
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For Russell

Who made it possible
Abstract

Introgression of genes from Rape to Wild turnip: Modeling transgene establishment in the New Zealand environment

By Toni E Jenkins

Introgression of genes from crops into ruderal populations is a multi-step process requiring sympatry, synchronous flowering, chromosomal compatibility, successful pollination and development of the zygote, germination, establishment and reproduction of hybrid progeny. The goal of this thesis was to generate data on as many steps in this process as possible and integrate them into a predictive statistical model to estimate the likelihood of successful introgression under a range of scenarios. Rape (Brassica napus) and wild turnip (B. rapa var. oleifera) were used as a model system. A homozygous dominant mutation in the rape genome conferring herbicide resistance provided a convenient marker for the study of introgression. Potential differences between wild turnip populations from a wide range of geographic locations in New Zealand were examined.

Hand pollination established the genetic compatibility of rape and wild turnip and a high potential for gene introgression from rape to wild turnip. Interspecific hybrids were easily generated using wild turnip as the maternal plant, with some minor differences between wild turnip populations. The frequency of successful hybridisation between the two species was higher on the lower raceme. However, the upper raceme produced more dormant interspecific hybrid seed.

Field trials, designed to imitate rare rape crop escapes into the ruderal environment, examined the ability of rare rape plants to pollinate wild turnip plants over four summers. At a ratio of 1 rape plant for every 400 wild turnip plants, the incidence of interspecific hybridisation was consistently low (<0.1 to 2.1% of total seed on wild turnip plants). There was a significant year effect with the first season producing significantly more seed and a greater frequency of interspecific hybrid progeny than the other years. The frequency of interspecific
hybrid progeny increases when the ratio of rape: wild turnip plant numbers increases.

The relative importance of anemophily and entomophily in the production of interspecific hybrids was examined. Wild turnip plants produced twice as many seeds with bee pollination relative to wind pollination. However, the frequency of interspecific hybrids under wind pollination was nearly twice that for bee pollination. Light reflectance patterns under UV light revealed a marked difference between wild turnip and rape flowers compared to near identical appearance under visible light. The data indicates that bees are able to distinguish between rape and wild turnip flowers and exhibit floral constancy when foraging among populations with these two species.

Hybrid survival in the seed bank, germination and seedling establishment in the field are important components of fitness. Seed banks established in the soil after the field trials described above germinated in subsequent spring seasons. The predominantly brassica weed populations were screened for herbicide resistance and the numbers of interspecific hybrids germinating compared to the original frequency in the field trial results. Frequency of interspecific hybrids was reduced in the populations compared to the original seed deposit. Seed with a known frequency of interspecific hybrid seed was sown in a separate trial, and the frequency of interspecific hybrids compared at 0, 4, 6, and 8 weeks after sowing. Poor germination resulted limited competition between seedlings, however the frequency of interspecific hybrids declined over time indicating low plant fitness. There were no significant population effects on any parameters tested.

Interspecific hybrids grown in a glasshouse were backcrossed to the parental species and selfed within the plant and within populations. Pollen from the interspecific hybrids was found to have markedly reduced fertility. Interspecific hybrid plants had low female fertility, with the majority (88%) of the pollinated flowers aborting the siliques. Of the remaining siliques, most (98%) had only one to three seeds per silique. Inheritance of the herbicide resistance gene was regular in backcrosses but highly skewed following self pollination with an excess of herbicide-sensitive progeny.
Production of a stochastic predictive model integrated the information acquired over the practical work phase of this thesis and utilised the capabilities of @risk, a new application of a risk analysis tool. The three outputs examined were the number of flowering plants resulting from backcrosses to rape and wild turnip and self pollination of the interspecific hybrid progeny. Five scenarios were modeled and all demonstrated the high likelihood of introgression failure in this system. In all scenarios, >75% of simulations resulted in no interspecific hybrid progeny surviving to flowering in the third generation. In all scenarios, and for all three outputs, the seed set on the interspecific hybrids of the second generation was the major factor that limited the number interspecific hybrid progeny surviving to flowering in the third generation.
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Chapter 1 Literature review

1.1 General Introduction

Hybridisation occurs between plants for which the interspecific barrier is incomplete (Grant 1981). As plants evolve the barriers between species become stronger (Ellstrand, 2003). Until the species barrier is complete, plants can exchange genes, and introgress new genes into their genetic inheritance. Plants may be closely related, but unable to be pollinated with each other's pollen (e.g. *Gilia transmontana*, *G. flavocinta*, and *G. malior* (Grant, 1964).

Hybridisation and gene flow have been recognized since Victorian times, with Darwin (1876) recording the effects of cross-fertilization amongst plants.

Introgression is defined as the stable integration of new genes from other individual plants, populations or species into a genome. Introgression can occur naturally, and may contribute to the evolution of new traits in species and of new species. Introgression has been used to transfer desirable traits into agricultural crops through traditional breeding.

Gene flow can occur from wild populations to crops and in the opposite direction (Ellstrand, 2003). Prior to the 1980s, gene flow was principally the concern of plant breeders, whose work concentrated on the use of separation distances to preserve genetic purity in seed crops. Since the introduction of transgenic plants, gene flow has become more important in the eyes of the public, including environmental activists and regulatory agencies, due to uncertainty about the effects of transgenes on wild populations (Kapteijns, 1993; Rogers and Parkes, 1995; Dale *et al.*, 2002). Gene flow from transgenic crops is perceived as a risk, and so risk analysis of transgenic crops includes assessment of the likelihood of introgression from the new crop to related crops and weeds (Ellstrand and Hoffman, 1990; Beckie *et al.*, 2003; Conner *et al.*, 2003). Control of gene flow through the reduction in pollen movement is usually achieved through either isolation zones or border planting of pollen traps (Staniland *et al.*, 2000). The movement of genes between species, and induced mutations in genetic material available from all species (through irradiation or
chemical mutagenesis) is not new to plant breeding. The wide range of genetic material unlimited by sexual compatibility barriers or scientist's ability to overcome those barriers, is new (Gepts, 2002).

As well as opportunities for the improvement of crops, this new approach to gene transfer is often perceived as presenting potential problems (Conner et al., 2003). These include: 1) that the transgenic plant will become an agricultural weed, with the modification making it more difficult to control, 2) that the transgenic plant will hybridise with wild relatives, creating weeds more difficult to control, or changing the composition of natural plant communities, 3) that these plants will contaminate conventionally bred crops of the same species, thereby reducing their value as genetic modification free, 4) that the genetically modified plants may present a direct risk to human health through toxic or allergenic effects, and finally 5) social or ethical concerns.

Genetic modification of food plants offers substantial new opportunities to crop breeders (Nap et al., 2003). Genetic modification allows the movement of genetic material between species (plants, animals or microorganisms) (Tiedje et al., 1989). As well as moving genetic material between species, genetic modification can silence particular genes or can be used to over-produce specific proteins (Ellstrand, 2003). Plants may be able to be used for the production of new compounds useful to the productivity of a crop plant (such as insecticidal proteins (Blackshaw et al., 1994)), or for the production of novel chemical compounds (such as palmitic acid from brassica plants), using the plant as a "solar-powered" chemical factory. Alternatively, plants can have their biochemical pathways altered in order to allow them to live in environments that would normally be considered toxic (such as soils with high heavy metal levels, Misra, and Gedamu, 1989), or to more efficiently utilise mineral resources (for example, plants modified to extract and retain gold from mine tailings, Robinson et al., 2003).

New technologies have often provoked fear in the general population, and genetic modification is no exception (Snow, 2002). The precision with which single genes can be transferred, therefore reducing the possibility of unexpected consequences, is seen by many people as risk reducing. Some
traits can be introduced to crop plants by more traditional breeding techniques, as well as by genetic modification (Preston and Rieger, 2000). Monitoring of transgenic herbicide-tolerant rape would be simpler than monitoring varieties with the same characteristics created with other technologies (Senior and Bavage, 2003). Opponents to genetic modification often describe the techniques as unnatural and with dangerous unknown consequences (Altieri, 2000).

An appropriate response to the discussion of the risks of genetic modification is to analyse the risks involved with the release of transgenic plants with controlled experiments (Raybould and Gray, 1993). These may be conducted in enclosed environments under containment conditions, or using non-transgenic, but genetically similar plants in field experiments.

1.2 Brassica and gene flow

Each crop presents a different risk profile depending on its life cycle and its mode of reproduction, and the presence or absence of crop and wild relatives with which it can exchange genetic material. Information of this type can be presented in literature reviews (e.g. Scheffler and Dale, 1994; Williamson and Fitter, 1996) or in 'botanical files' such as that created by Landcare (Newstrom et al., 2003). Brassica presents a potentially high risk case due to the ability of the family to produce interspecific hybrids, and the presence of native, wild and cultivated forms (Chèvre et al., 1996, 1997, 1998).

There are some native Brassica in New Zealand, but they are not members of the same tribe as rape and wild turnip (Bourdōt et al., 1999). The Brassica tribe members that are present in New Zealand are not all present in the oilseed rape growing areas, but if herbicide resistant forage Brassica are developed then more weeds will be exposed to the possibility of gene introgression.

Brassicas are a large group of plants that contain several important agronomic crops (Table 1). These include some vegetables (i.e. cabbage, cauliflower broccoli and kohl rabi), some animal feed crops (i.e. swede, kale and turnip) and some oil crops most notably in this case Canola or oilseed rape. The Brassica family also contains some ornamental plants (e.g. ornamental kale,
alyssum) and some weeds (e.g. wall cress (*Arabidopsis thaliana*), winter cress (*Barbarea* sp.)). The main Brassica weeds are wild turnip (*B. rapa*), wild radish (*Raphanus raphanistrum*) and hoary mustard (*Hirschfeldia incana*). It is possible to make interspecific and intergeneric crosses within the Brassica tribe (Brown *et al.*, 1991), though some of these crosses need significant scientific intervention (Scheffler and Dale, 1994). The resulting hybrids have varying levels of fertility. Some are able to backcross and produce progeny while others are male sterile, female sterile or are completely sterile (Dale, 1992, 1994; Scheffler and Dale 1994). The importance of hybrids between rape and wild turnip in the ruderal environment, and potentially with transgenes has been reviewed (Gliddon, 1994). Rape has no initial seed dormancy, while some of the weeds in the Brassica tribe have strong seed dormancies. Some interspecific hybrids may have increased seed persistence and may inherit the characteristics of wild turnip seed (Linder and Schmitt, 1995).

Table 1. Brassica present in New Zealand

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific species name</th>
<th>Varieties present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turnip</td>
<td><em>Brassica rapa</em></td>
<td>rapa, glabra, oleifera, chinensis</td>
</tr>
<tr>
<td>Rape, oilseed rape, swede</td>
<td><em>Brassica napus</em></td>
<td>napus, napobrassica</td>
</tr>
<tr>
<td>Cabbage, cauliflower, broccoli, Brussels sprouts, kale</td>
<td><em>Brassica oleracea</em></td>
<td>gongylodes, acephala</td>
</tr>
<tr>
<td>Indian or brown mustard</td>
<td><em>Brassica juncea</em></td>
<td>juncea</td>
</tr>
<tr>
<td>Black mustard</td>
<td><em>Brassica nigra</em></td>
<td></td>
</tr>
<tr>
<td>Mediterranean mustard</td>
<td><em>Brassica</em></td>
<td><em>tournefortii</em></td>
</tr>
</tbody>
</table>

Brassicas grow throughout New Zealand and are used for both animal and human consumption and occur as weeds. There are both native and introduced brassicas, though only introduced ones are used in the agricultural environment for cropping (Bourdöt *et al.*, 1999). Wild turnip, which may be one of four varieties present in New Zealand (Heenan *et al.*, 2004) may be from crop
escapes rather than the genuine wild turnip as described by Crouch et al. (1995) and Heenan et al. (2003). The most common wild turnip weed variety is *B. rapa* ssp. *oleifera* which persists in the ruderal environment (Heenan et al., 2003). Rape is cultivated for two end products; 1) as a stock feed, used before flowering occurs and S-methyl cystine sulphoxide levels become toxic, and 2) as an oilseed crop. To produce seeds for re-seeding or oil production rape must be allowed to flower, and it is the movement of pollen during flowering that presents the greatest risk of gene flow.

Turnip and rape are closely related botanically as described in the next section, and flower at similar times in the agricultural habitat. This increases the potential for genes to move via pollen from one species to another. Rape and wild turnip (*B. rapa*) have been shown to hybridise. Gene flow from rape could conceivably result in creating genetically modified wild turnip weeds, if established wild turnip populations flower near transgenic oilseed rape crops, and pollen is moved between the species. Some data suggest there are no ‘costs’ associated with the introgression of transgenes (Snow et al., 1999).

Rape and wild turnip are considered to be a high risk combination, due to them growing in the same environmental conditions, having sympatric flowering, and being capable of forming interspecific hybrids.

Wild turnip (*B. rapa* var. *oleifera*) is an annual, again with a lax rosette, but in this case the leaves are bristly. The floral structure is the same height (~1.5 m), and similar in structure, but with open flowers overtopping the buds. The reddish brown to black seeds produced by both species are indistinguishable. It is possible to pick the two species apart by their morphology, but the differences are subtle, and can be obscured by variations in their variety or genotype (Heenan et al., 2003).

Rape (*Brassica napus* var. *napus*) is an annual or biennial herb which forms a loose rosette, before producing a floral structure which may be up to 1.5m in height. The leaves are glaucous, sometimes with bristles on the veins. The flowers are bright yellow, with the unopened buds held above the open flowers. The position of the buds relative to the flowers is an important distinguishing characteristic differentiating rape from wild turnip. It is however not reliable, and
flow cytometry is the only truly accurate way to identify the species (Heenan et al., 2003). Seeds are produced in siliques, with up to 32 seeds in each silique. Seeds are spherical, reddish brown to black, and 1.5-2.5 mm in diameter. Rape has poor persistence in the ruderal environment (Sweet et al., 1999; Crawley et al., 2001; Simard et al., 2002) and is not considered a significant weed (Salisbury, 2000).

Rape is a plant that is relatively easy to modify (Senior and Dale, 2002) using genetic engineering techniques such as Agrobacterium mediated gene transfer or micro ballistics, with transformation efficiencies of up to 25% (Cardoza and Stewart, 2003). Genetically modified oilseed rape (Brassica napus) is a potentially useful agricultural tool in New Zealand, as rape is a useful break crop in cereal rotations. It can be very difficult to control dicotyledonous weeds in rape crops, especially if the weeds are members of the Brassica family (Bourdôt et al., 1999). Genetically modified rape that is resistant to a general herbicide such as glyphosate would be a useful agronomic tool for weed control as both cereal volunteers, monocotyledonous and dicotyledonous weeds could all be effectively and efficiently eliminated from the rape crop. Herbicide resistance would also be useful in the establishment phase of pasture crops.

1.3 History of interspecific hybridisation in Brassica

The production of interspecific hybrids has been used to discover and confirm relationships between the members of the brassica tribe. Evidence of the relative weakness of the species 'barrier' was reported in the 1800s, and indicated that brassica species might be closely related to each other. The first recorded artificial cross was that of swede (B. napus) and turnip (B. campestris) by Herbert in 1834, as reported in the paper by Kajanús (1917). Observations of natural interspecific hybrids between brassica species were also made by Darwin (1876), who described 'bastard plants' of intermediate morphology forming where two brassica species were grown in close proximity.

Further work was carried out by Sutton (1908) on the ability of different member of the brassicas to interbreed. He worked with four species, being B. napus, B. oleracea, B. campestris (syn. rapa) and B. rapa. His results demonstrated that
interspecific hybrids could be produced from a range of crosses, but most importantly for this present thesis, that it was possible to produce interspecific hybrids from *B. napus* and *B. rapa*. In his paper he noted the offspring were mainly sterile, “while others unfortunately died off in the winter before the question of their fertility or sterility could be ascertained”. Kajanus (1917) produced 437 seeds from 38 pollinations using turnip as the male parent, and 13 seeds from 19 pollinations with the reciprocal cross. This is in direct contrast to most subsequent workers who find the rape is most successful as the pollen parent in the production of F1 hybrids.

An extensive study by Nelson (1927) sought to describe the fertility of various commercially produced hybrid brassicas with a view to commercial seed production. He confirmed the work of Sutton (1908), showing that the turnip x rape cross was more successful with turnip as the female parent, and noted that this was possibly due to the chromosomal numbers. The observation was made that interspecific crosses were more successful with the plant with the larger chromosome number (in this case rape with 2n=36) being used as the male parent. His turnip x rape crosses usually produced seed, with the resulting hybrid plants having less vigour than the pure rape. The hybrids produced with the rape as the pollen parent were able to be selfed, with a small number of pods produced, and 1-2 seeds forming in the successful pollinations. Backcrossed hybrids were found to be “more or less completely fertile”.

In the late 1920s, interspecific hybridisation was used to explain the phylogenetic relationships between the species of the Brassica genus. Based on such results, Pearson (1928) reduced the number of Brassica species from 33 (after Schulz 1919) to four. A high number of the crosses made between rape and turnip formed siliques (up to 100%) and 7-11 seeds were formed in each silique when rape was used as the female parent. When turnip was used as the female parent there were many siliques formed, but all the floral structures were subsequently lost to soft rot disease.

One of the most important papers describing the genetic relationships between Brassica species was published by U (1935). His paper described the hybrids obtained between *B. oleracea* and *B campestris* followed by chromosome
doubling as experimentally formed *B. napus*. The cross between the *B. napus* and either of the two progenitor species is similar to a backcross, and to some extent provides an explanation for the low level of interspecific barriers to hybridisation. This paper established what is now known as the triangle of U (Figure 1). This shows rape as an amphidiploid derived from *B. rapa* (turnip) and *B. oleracea* (cabbage). The relationship between rape and wild turnip has been confirmed through artificial synthesis of rape by Downey et al. (1975) and Olsson and Ellerstrom (1980). Cytological evidence indicates *B. rapa*, *B. oleracea* and *B. nigra* are secondary polyploids deriving from a common ancestor with a chromosome number of *x*=6. Interchromosomal similarities therefore exist throughout the ‘Triangle of U’, enhancing the possibility of hybridisation between the species. The crossing of the tetraploid rape and the diploid turnip results in triploid progeny, which are highly likely to be infertile.

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Figure 1. The Triangle of U showing relationships between members of the brassica tribe.
The first experimental crossing of *B. rapa* and *B. napus* in the field was conducted by U and Nagamatsu (1933). This was part of the cytological studies conducted by U. When *B. rapa* was used as the female parent, no hybrids were produced on the 3520 plants examined. Nineteen different rape genotypes were used. When *B. rapa* was used as the male parent, 6349 plants were examined and 266 hybrids reported. These plants were described as hybrids on the basis of their morphology, which was intermediate to the two species. No F₂ or backcross plants were reported.

Experiments in interspecific hybridisation of brassica were first conducted in New Zealand by Calder (1937). Calder established through hand pollination that the biotypes present in New Zealand were able to produce interspecific hybrids between rape and turnip, with 228 seeds obtained from 101 pollinations. He also grew rape and turnip plants in close association, and the seed obtained was grown to determine the degree of natural interspecific hybridisation. All seed obtained from the rape plants resembled rape, while turnip plants open-pollinated in the presence of rape did produce turnip x rape hybrids, and the turnip plant was also noted to be self-infertile. Calder (1937) reported 25 hybrids produced from the cross when *B. rapa* was used as the female parent in the field, in contrast to the complete lack of hybrids reported by U and Nagamatsu (1933).

Olsson (1949) produced 4294 interspecific hybrid seeds from 1225 pollinations in her study of the hybridisation ability of different Brassica species. Mizushima (1950) produced rape x turnip hybrids in the course of his work on karyogenetic studies of hybrids in Brassicaceae. There is no record of the ease or frequency of interspecific hybrid production.

Nwankiti (1971) produced at least one rape x turnip hybrid to use in backcross studies, as part of a project to establish new chromosomal numbers such as 2n=32. This plant was successfully used to produce F₂ hybrids and backcross progeny (crossing back to turnip).

Heyn (1977) conducted an in depth study of unreduced gametes in interspecific crosses in Brassicaceae. It was found that rape and turnip crosses set seed readily, whether on emasculated and hand pollinated flowers, or using male...
sterile plants of rape with fertile turnip in mixed stands. About 70% of the flowers set seed, with 7 – 14 seeds in each siliquae. This researcher discussed the production of non-hybrid matromorphic seed, which was first described by Noguchi (1928) and also reported by Röbbelen (1966), Tokumasu (1965, 1970), Mackay (1973) and Eenink (1975). These most probably arise from unreduced gametes, and are described as being of "rather common occurrence".

1.4 Interspecific hybridisation between *B. napus* and *B. rapa* for the purpose of classical plant breeding

Interspecific hybrids have been used to create new types of vegetables, or to move useful genes between species. Interspecific hybridisation is relatively easy in Brassica as there are weak interspecific barriers between some species.

In an example of the use of hand pollination to transfer desirable characteristics of one species into another, Lammerink (1970) transferred resistance to a particular race of clubroot from *B. campestris* to *B. napus*. Lammerink (1970) states "There were no major fertility barriers between the two species", and goes on to note that "F₃ progenies were morphologically identical to and fully inter-fertile with ...*B. napus*. This rapid gene introgression has obvious implications for gene flow and introgression from transgenic *B. napus*.

Mackay (1973) attempted field production of hybrids of rape and turnip. The hybrids were felt to offer opportunities for a new forage rape type. Production of the hybrid needed to be simple and preferably possible without human intervention, and so rape and turnip plant pairs were placed in a cage and allowed to pollinate 'naturally' in cages using blowflies as pollen vectors. The self incompatibility of turnips was used to encourage interspecific hybridisation. From each turnip plant, mean seed yields of 5.2 ± 4.0g were obtained, and the nine plants had frequencies of hybrid progeny varying between 30.1 and 96.8%.

Johnston (1974) describes the production of rape x turnip hybrids, developed for the purpose of transferring the resistance of turnip to the disease *Plasmodiophora brassicae* and subsequently backcrossed to rape. The
procedure produced the fully fertile artificial amphiploid species *B. napocampestris* (2n = 58 AAAACC), through the application of colchicine to the cotyledons of rape x turnip hybrid seedlings. Within two generations, chromosome counts of the BC2 plants generated from the *B. napocampestris* plants had returned to the rape chromosome count. Although the production of *B. napocampestris* was highly artificial, the rapid return of the disease resistant backcross progeny to the parental chromosome counts has implications for the introgression of transgenes into the wild turnip population.

Namai (1977) produced forty-four sesquidiploid F1 plants from the cross between *B. napus* and *B. campestris*, in an attempt to transfer economic characteristics between the two species (as well as between other brassica species). Breeding on from the F1 generation plants produced the observation that in the next generation there were plants that closely resembled *B. campestris* which were obtained from back-crossing with campestris or by open pollination. Namai goes on to state that translocation of chromosomes between the A genome and C genome must have taken place during the meiotic division in the amphihaploids.

Beversdorf *et al.* (1980) crossed *B. campestris* and *B. napus* in an attempt to transfer triazine resistance from the *B. campestris* to the *B. napus*. More than five hundred hand pollinations were made resulting in 52 viable seeds. Fifty of these germinated, and where *B. campestris* was used as the female parent all fifteen hybrid progeny inherited the resistance trait.

1.5 Interspecific Brassica hybridisation studies investigating risk analysis for transgenic plants

Bing *et al.* (1991) examined the ability of the cultivated oilseeds *B. napus*, *B. campestris* and *B. juncea* to exchange genetic material. All previous researchers were interested in the genetic relationships between brassicas or with the transfer of desirable genetic traits between species. Bing *et al.* (1991) produced the first published paper examining the risks associated with interspecific transgenic gene flow. The reciprocal ability of *B. napus* and *B. campestris* to produce interspecific hybrid progeny was assessed under both
hand pollination and open pollination in the field (Bing et al., 1991). These results establish there is no significant barrier preventing hybrid production between these two species. When *B. campestris* was used as the female parent, it was half as successful as the reciprocal cross, but still produced a Controlled Interspecific Cross Compatibility (= number of hybrid developed x 100/number of buds pollinated) of 934. The open pollinated field trials generated substantially different numbers, though the trends were similar, with *B. napus* being the most successful female parent (Natural Interspecific Cross Compatibility = 2.66; as opposed to *B. campestris* x *B. napus* = 0.99. Bing et al. (1991) reported reduced pollen fertility in the interspecific hybrid plants, however the values for the *B. napus* x *B. campestris* plants are not shown. Further work by Bing et al. (1996) reported very similar results with hybrids between *B. napus* and *B. campestris* produced under open pollination in Canada.

Brown and Brown (1996) used hand and field pollinations to ascertain the likelihood of transgene escape from cultivated to related weedy brassicas. Detailed dissection of the floral structures at different stages of fertilisation showed that the interspecific cross resulted in pollen grains that successfully germinated and penetrated the stigma, that pollen tubes were detected in the ovary and the pollen tubes penetrated the ovule.

Hand pollination experiments show the potential for interspecific hybridisation in artificial conditions. The results obtained may not transfer directly into the likelihood of interspecific hybridisation in the field. Hand pollinations usually use bud pollinations, in order to ensure there are no other pollen grains present on the stigma apart from those introduced deliberately. However bud pollination can also be used to overcome incompatibility (Cabin et al., 1996), as the compounds responsible for incompatibility are accumulated in the flower after the compounds that stimulate pollen germination (Shivanna et al., 1978). Brown and Brown (1996) demonstrated that risk assessment of gene flow cannot be performed using bud pollinations alone. Bud pollinations not only allow the recovery of interspecific hybrids that would otherwise not develop under open pollinated field conditions, but such controlled hand pollinations do not normally take into account the possibility of interspecific pollen competition.
on the stigma and in the style. Interspecific pollination in the natural field environment must by its very nature occur in mixed species stands, where it is highly likely mixed pollen will be deposited on the stigma. Hauser et al. (1997) demonstrated that mixed pollinations can result in preferential exclusion of the interspecific pollen. Other barriers to gene flow also exist, including synchrony of flowering, which can be overcome in field experiments, but should be assessed by observation of natural mixed stands of the two species being considered, and physical proximity (sympathy).

In a study conducted with New Zealand biotypes Palmer (1962) reported many hybrids produced in the field between *B. rapa* and *B. napus*. He used 2 rape genotypes, and both wild and cultivated turnips, but the presented results do not distinguish between the wild and cultivated turnips. The success of his crosses depended on the relative proportions of rape to wild turnip. When the self incompatible turnip flowered in the presence of other turnip plants there was preferential use of turnip pollen to set turnip progeny. However, in the absence of compatible turnip pollen, turnip plants were able to utilise rape pollen for the successful production of interspecific hybrid progeny. There may be some variation between rape genotypes in their ability to produce interspecific hybrids (Baranger et al., 1995).

Scott and Wilkinson (1999) and Wilkinson et al. (2000 and 2003) also studied the establishment of *B. napus* and *B. rapa* interspecific hybrids in natural situations in the United Kingdom. The first publication established a low level of sympatry between the two species (0.6 – 0.7%), suggesting that mixed stands will be rare Scott and Wilkinson (1999). It is also reported that rape populations suffer from a rapid decline in numbers over three years. Wilkinson et al. (2000) used satellites in 1998 to find sympatric populations of *B. napus* and *B. rapa* over 15 000 km² of south-east England, and discovered two sympatric populations. In the next year every plant of these populations was screened for hybrid status, and 1/505 plants was found to be an interspecific hybrid. A report on the national likelihood of interspecific hybrids of *B. napus* and *B. rapa* was published in 2003 (Wilkinson et al., 2003). Using a wide range of sources of information including "population surveys, remote sensing, pollen dispersal profiles, herbarium data, local Floras and other floristic databases", they
estimate that 32,000 hybrids form annually in waterside populations in the United Kingdom. Hybrids are still described as rare by Stace (1991) even though they have been presumably present each year. This suggests that they do not survive or thrive in the natural environment.

1.6 Danish studies of interspecific hybrids of *B. napus* and *B. rapa*

In 1994 a Danish research group began an intensive and extensive study of the ability of *B. napus* to transfer genes to *B. rapa* as a risk assessment for the release of transgenic *B. napus*. Their first paper (Jørgensen and Andersen 1994) discussed the previous literature and stated the "crossibility between oilseed rape and *B. campestris* is controversial". The ability of plant breeders to cross the two species through hand pollination is not in dispute, but natural interspecific crosses were described as rare. Results from PROSAMO (Dale et al., 1993) are quoted which state neither *B. oleracea* or *B. rapa* can be fertilised with *B. napus* pollen (Jørgensen and Andersen 1994). Both the Bing et al. (1991) paper and the Palmer (1962) paper are cited as examples where the two species have been recorded as naturally crossing. Jørgensen and Andersen examined hybridisation frequency in three different experiments. Firstly, five Danish populations of wild turnip and two varieties of spring sown oilseed rape were sown at a 1:1 species mixture; 9-29% of the progeny were hybrids. Secondly, isolated individual *B. campestris* plants from four Danish populations were planted in 2 varieties of *B. napus*, interspecific hybrids comprised between 56 and 93% of the progeny. Thirdly, a natural weedy population of twenty *B. campestris* plants growing within a field of winter oilseed rape produced 60% hybrid progeny. They concluded that transgenes could be dispersed from oilseed rape to *B. campestris*.

In a subsequent paper, Jørgensen et al. (1996) demonstrated that backcross progeny could be produced in the field. Hybrids had reduced pollen fertility (35% on average) and produced 102 seeds/hybrid through backcrossing to the parental species (n=66). Hybrid status was shown through the inheritance of 17 oilseed rape specific markers, and in separate experiment, through the inheritance of herbicide resistance.
The Danish research group then published a study of the inheritance of RAPD markers in backcross progeny (Mikkelsen et al., 1996b). Interspecific hybrids produced on *B. napus* females, pollinated with *B. campestris* [sic] were subsequently used as females and backcrossed to *B. campestris*. This report describes much lower seed set in the backcross with 0.7 seeds set per pollination (interspecific hybridisation resulted in 9.8 seeds set per pollination). Transfer of markers to the backcross generation was reported as 26% to 91%. Investigation of 33 markers in different parts of the chromosomes showed all could be transferred to *B. campestris*. This paper dismisses the hypothetical existence of ‘safe’ integration sites.

There had been some speculation that due to the lack of homologue chromosomes to pair at meiosis in interspecific hybrids of rape (AACC) and wild turnip (AA) for the C genomes, that this might present a ‘safe’ integration site for transgenes (Chen et al., 1990; McGrath and Quiros, 1990). Metz et al. (1997) studied the transmission of transgenes through four generations of backcrosses, and found approximately 10% of offspring carried the transgene in the BC3 and BC4 generations. Transmission varied between offspring, and the differences were attributed to transgenes being integrated into a C-genome chromosome (Metz et al., 1997). This conclusion is disputed by Tomiuk et al. (2000) as A and C chromosomes are differentiated only by chromosomal rearrangements – e.g. translocation, inversion and gene duplication, and are homeologous, thereby allowing recombination. Lu et al. (2002) examined the transmission of genes on both chromosomes using three models, and concluded that gene transmission would be reduced if genes were on the C-chromosome.

Landbo et al. (1996) expanded the data presented in Jørgensen and Andersen (1994) with more data from natural populations of *B. campestris*. Many of the populations examined contained no hybrids, in contrast to the results presented in Jørgensen and Andersen (1994). Landbo et al. discussed this disparity, and speculate that it could be caused by differences in the *B. campestris* populations, by the spatial structure of the *B. campestris* and *B. napus* populations, and by the synchrony in flowering times. The Danish *B. campestris* has a strong seed dormancy, and the methods of breaking seed
dormancy varied between Jørgensen and Andersen (1994) and Landbo et al. (1996). This effect can change the reported frequencies of hybrid progeny, depending on whether the percentage is calculated against the total seeds harvested, the number of seeds germinated or the number of seedlings surviving to testing.

The germination behaviour of the parental species and the interspecific hybrids is another consideration important for risk assessment. Landbo and Jørgensen (1997) found cultivated *B. napus* seed had no dormancy, while *B. campestris* seeds varied according to the colour of the seeds, with light coloured seeds being non-dormant or dead compared to the dark coloured fraction. There was a great deal of variation in the requirement for dormancy breaking treatment in the seed produced by a single *B. campestris* plant, as well as by plants arising from a single population. Reciprocal cross interspecific hybrids all showed the dormancy patterns of *B. napus*, i.e. no dormancy at all. The seeds of these hybrids were shown to resemble the dormancy behaviour of *B. campestris*, thereby increasing the likelihood the backcross generation could survive and procreate for many years. Adler et al. (1993) found canola type *B. rapa* had higher germination frequencies than wild *B. rapa* populations, and that hybrid germination (of the two *B. rapa* types) was more similar to maternal germination patterns. Seed mortality and dormancy are often strongly influenced by the seed coat, which is derived from maternal tissue. Hybrids generated from maternal wild type *B. rapa* plants were more likely to persist in the natural environment, due to their ability to remain dormant in the seed bank (Adler et al., 1993).

Once an interspecific hybrid has formed, the fitness of the F₁ and F₂ progeny is critical for subsequent gene introgression. F₁ hybrids were found to be intermediate in fitness between their *B. napus* and *B. rapa* parents, and differences were detected between offspring produced by individual parental plants, populations and varieties for some of the fitness components scored (Hauser et al. 1998a). Fitness was assessed by examining the number of flowers that developed into pods, fully developed seeds per pod, proportion of fully developed seeds, survival of seedlings, pods per offspring plant and seeds per pod on offspring plants. The number of seeds produced per F₁ interspecific
hybrid plant was intermediate to the number of seeds produced by the parental species. This is in contrast to the results of Jørgensen and Andersen (1994) and Mikkelsen et al. (1996 ab), and the review by Scheffler and Dale (1994). Hauser et al. (1998a) speculated that this could be due to the reduced planting density in this experiment. The fitness of F2 and backcross plants was subsequently examined (Hauser et al. 1998b) with these F2 plants and backcross plants being significantly less fit than their parents or the F1 generation. F2 plants were the least fit of any examined. Significant differences were detected between B. campestris populations, B. napus varieties and parental plants on the fitness of the subsequent generations (Hauser et al., 1998 a and b). It is suggested that risk assessment of transgenic plants should be done with local wild populations that might be transgene recipients. Nevertheless, even though F2 interspecific hybrid plants had low fitness, they were still as fit as some of the B. rapa plants (Hauser et al., 1998b).

Although all these experiments suggest that introgression is likely to occur, and may indeed already have occurred, the results have been collected from experimental plots, while introgression may not occur in the natural environment. Hansen et al. (2001) examined a natural population of brassica plants which included B. napus and B. rapa like plants, and examined the species-specific AFLP-markers to assign parentage. They found nearly half of the plants collected from their plot possessed introgressed chromosome regions. Most of the weedy population appeared to be resemble second backcross generation (Hansen et al. 2001).

1.7 Vectors for pollen distribution

The movement of pollen between rape and wild turnip must be achieved by a vector. In the case of rape, Timmons et al. (1995) have suggested that rape is pollinated by three mechanisms 1) insects, 2) wind and 3) mechanical pollination (where two flowers hit each other). Wild turnip is said to be wholly insect pollinated (Holm, 1997), and so the vector for the cross pollination of rape and wild turnip would logically be expected to be insects, that being the vector they have in common. Much has been made of the possibility of pollen travelling long distances by wind (e.g. McCartney and Lacey, 1991; Timmons et
al., 1995), but it has not been determined whether this pollen would survive for long periods in the environment (Rao et al., 1992) successfully germinate on normal (unemasculated) flowers, and whether it would overcome interspecific barriers and produce seeds.

Honeybees are the major pollinator responsible for insect vectored pollen movement. Exclusion of honeybees has been shown to slightly reduce the productivity of rape plants (Persson, 1956), and the abundant nectar produced by the flower is highly attractive to honeybees (Free and Nuttall, 1968). The distance that honeybees (Apis mellifera) can carry pollen has been recorded at approximately 6km, with individuals traveling up to 3 km from the hive on foraging trips (Ramsay et al., 1999). Honeybees have also been shown to retain viable sticky Brassica napus pollen after returning to the hive and removing/cleaning the visible pollen grains (Ramasy et al., 1999), therefore spreading pollen on subsequent trips, possibly between species. Honeybees are thought to remain loyal to plant species on each foraging trip (Proctor et al., 1996), to improve the effectiveness of pollen packing in the corbiculae (Zahavi et al., 1983).

Honeybees have the potential to be important vectors for pollen transfer between brassica species. The flowers are highly attractive to them (Fries and Stark, 1983), and the two species appear highly similar in colour and structure, indicating honeybees may move freely between them. Alternatively, wind pollination could be the most important vector of pollen for interspecific pollination. Rape fields release large quantities of pollen (Williams, 1984), which can be carried 1.5km (Timmons et al., 1985). The effectiveness of pollen traps and barren zones (Morris et al., 1994) depends on the relative importance of bees in gene flow.

1.8 Fitness of interspecific hybrids of B. napus and B. rapa

Breeding success is not the only criteria that need to be examined to assess the likely introgression of genes into wild populations. Various measures can be taken as estimates of plant fitness and invasiveness. Seed survival is an important component of fitness (Chadoeuf et al., 1998). Plant fitness includes
the ability to grow, which can be measured simply in terms of dry matter accumulation, and in records of floral timing and volume. The ability of the hybrid to grow however only measures a very short-term measure of the success of the plant. A successful plant must be able to reproduce, and in the case of a new hybrid, it is most likely to be successful if it is able to backcross to its parents and to self either within the plant, or within a potential hybrid population. For gene introgression backcrossing to the parents is essential.

Physiological fitness also effects the rate of introgression, and in Hauser and Østergård (1999) there is a report of a mal-adjustment of the interspecific hybrid seeds of *B. napus* and *B. rapa*, where seeds germinate in the siliques thereby reducing dispersal and increasing the chances of mortality due to desiccation. Vivipary has been reported in *B. napus* and *B. rapa* interspecific crosses before (Calder, 1937; Nishiyama et al., 1991) and is considered to be deleterious to seed survival due to increased likelihood of disease, desiccation and no possibility of long term survival in the seed bank.

There is a frequency linked fitness element to the success of the interspecific hybrids. The interspecific hybrids studied by Hauser et al. (2003) are more fit than the *B. rapa* parent where fitness is measured by seed production. Pertl et al. (2002) and Hauser et al. (2003) describe the effects of frequency of species on the fitness of species. Both species and backcross plants are more fit in pure stands, while *F₁* plants produced more seeds when planted in mixed stands and fewer in pure stands. These results may be due to vegetative and reproductive interactions. *F₁* male fitness is very low (Pertl et al., 2002; Hauser et al., 2003), but *F₁* female fitness can be high depending on frequency of hybrids in a population. The likelihood of introgression depends in part on the *F₁* frequency: where *F₁* frequency is low among a population of *B. rapa* plants the likelihood of introgression is increased.

1.9 Modeling for gene flow and risk assessments

Transgenic oilseed rape, as one of the early transgenic crops to be commercialized, has been the subject of extensive research. This has resulted in a substantial body of data which can be used as the basis for predictive
modeling. Information includes the role of pollinators (Cresswell, 1995) and gene flow between plants (Kwak and Velterop, 2001). This can then be used to predict population dynamics, in this case of the release of a new variety. The need for computer models simulating the introgression process was recognised by Darmency (1994).

Ecological models are used to describe the interactions between an organism and its environment, and in the case of the release of transgenic varieties, the numbers of individuals in the population of the organism under study (Gillman and Hails, 1997). The expression of the population dynamics resulting from the release of the transgenic variety can be expressed in a mathematical equation, which allows manipulation of the factors in the model, and may make apparent any unassigned variation, thereby allowing improvement of the model.

Release of transgenic plants has an element of risk associated with it, as many plants have seed that is widely dispersed, or small, and so in many cases once the plant is released, it is unlikely that all individuals will be able to be recalled. It is better if more of the risks of the release can be studied through small experiments, possibly with model plants such as mutants, and these can then be extrapolated through the use of ecological models. The actual environmental risk can then be substantially reduced.

Crawley et al. (1993) used a simple model to describe the variables affecting the establishment and performance of transgenic plants over time. More sophisticated models have been developed by Davis et al. (1999) to explain the dispersal of transgenes in a stochastic environment. Cresswell et al. (1995) used the modeling approach to study the dispersal of pollen grains when moved by honey-bees and bumblebees, which is a part of the dispersal of transgenes from an oil-seed rape crop.

1.10 Goals and objectives of this thesis

The overall goal of this thesis was to investigate the likelihood of introgression of genes from rape to wild turnip in the New Zealand context.

To achieve this goal the following objectives were established:
To ascertain whether New Zealand isolates of *B. rapa* were able to form interspecific hybrids with *B. napus*. Hand pollination was used to maximize the likelihood of interspecific hybrid formation (Chapter 2). A seedling screening method was established, and its effectiveness confirmed with flow cytometry.

To examine the rate of interspecific hybrid formation in the field, *B. rapa* populations and *B. napus* resistant to chlorsulfuron were planted, and the seed screened for interspecific hybrids (Chapter 3).

To examine the relative importance of bee and wind pollination to the formation of interspecific hybrids, a glasshouse trial was established with a 1:1 mix of New Zealand *B. rapa* populations, and chlorsulfuron-resistant *B. napus*. A beehive was maintained in one, and a large fan used to simulate wind in the other. Seed produced was screened for the presence and frequency of interspecific hybrids (Chapter 4).

The ability of interspecific hybrids to survive in the seed bank, and survive germination and establishment was examined using the field trial sites established during experiments for Chapter 3, and also by planting known mixtures of *B. rapa* and interspecific hybrids of *B. rapa* and *B. napus*. Spraying seedling swards with chlorsulfuron resulted in the survival of resistant interspecific hybrids, allowing study of survival. Records were also kept of their ability to set seed (Chapter 5).

Transmission of the herbicide resistance gene, and fertility of the interspecific hybrids are important factors in their successful establishment in the ruderal environment. These factors were examined using hand pollination of interspecific hybrids from each of five New Zealand *B. rapa* populations. Each maternal plant was selfed with its own pollen, with pollen from another plant sourced from the same population and backcrossed to wild type rape and wild turnip. Any seed set was screened for herbicide resistance to ascertain gene transmission patterns (Chapter 6).

All the relevant information gathered from these experiments, and from other experiments conducted overseas will be used to generate a statistical model, which will be used to find the most appropriate timings and methods of control.
to reduce the introgression of transgenes from rape crops into wild turnip populations (Chapter 7).
Chapter 2: Population variability in wild turnip (Brassica rapa var. oleifera DC) for interspecific hybridisation with herbicide-resistant rape (Brassica napus L.) pollen¹

2.1 Introduction

The environmental release of transgenic crops with novel traits such as herbicide resistance presents a possible risk — that transgenes will introgress in populations of related weed species and result in enhanced fitness, survival, and spread of weeds (Conner et al. 2003). Rape (Brassica napus L.) is a crop at the forefront of transgenic development internationally, with commercial release of transgenic cultivars with herbicide resistance (to glyphosate, phosphinotricin, or bromoxnil), altered oil content, and male sterility/fertility restoration system for hybrid seed production (Nap et al. 2003). The Brassicaceae family is represented in New Zealand by at least 108 entities given specific, subspecific or varietal rank, of which 29 are indigenous, 69 are fully naturalised, and 10 have casual status (Webb et al. 1988; Parsons et al. 1998). None of the indigenous species are closely related to rape and of the introduced species, the one most likely to hybridise with rape is wild turnip (Brassica rapa var. oleifera DC) (Bourdôt et al. 1999). Note — wild turnip, formerly known as Brassica rapa subsp. sylvestris (L.) Janchen in New Zealand, is now refereed to as Brassica rapa var. oleifera DC (Heenan et al. 2004).

The ability of Brassica species to form interspecific hybrids has been well recognised for many years (e.g., Darwin 1876), with Sutton (1908) reporting

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"mongrel plants" abounded when brassicas of differing species were planted in close proximity. The deliberate interspecific hybridisation of *Brassica* species has occurred throughout the twentieth century for a range of purposes. Hand pollination has been used to study the taxonomic relationships (Pearson 1928; Sinskaia 1928; U 1935), to develop new breeding lines with valuable traits such as disease resistance (e.g., Gowers 1982), and to assess the risk of gene escape from transgenic crops (e.g., Bing *et al.* 1991; Kerlan *et al.* 1993; Wilkinson *et al.* 2000). A recent review of the literature listed 47 reports of rape × turnip and turnip × rape crosses from hand pollination, and only two of these studies failed to produce hybrid seed (Bourdôt *et al.* 1999). The reported frequencies of interspecific hybridisation can vary markedly between different studies. This can be attributed to differences in the experimental methods, the motivation for each investigation, manner in which data is recorded, and the specific genotypes used in each study (Bourdôt *et al.* 1999).

As a basis for risk assessment for the release of transgenic rape plants in New Zealand, this study investigated the potential for pollen-mediated gene flow from rape to wild turnip. Interspecific hybridisation between a non-transgenic herbicide-resistant rape (AACC; 2n = 4x = 38) and six New Zealand populations of wild turnip (AA; 2n = 2x = 20) is examined through hand pollination. Experiments were conducted in 2 years and investigated differences between rape pollen donor individuals, maternal wild turnip individuals, wild turnip populations, and early and late flowers as represented by the upper and lower racemes of wild turnip.

2.2 Materials and methods

2.2.1 Plant material

Seeds from six populations of wild turnip (*Brassica rapa* var. *oleifera* DC) were collected from throughout the southern 950 km of New Zealand (Table 2). The non-transgenic rape line (30a, developed from CrGC#5) was homozygous at a single locus for a dominant mutation conferring resistance to the herbicide chlorsulfuron (Conner *et al.* 1994).
Table 2. Origin and habitat description for wild turnip populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Massey, Palmerston</td>
<td>40°22'642&quot; S, 175°36'427&quot; E</td>
<td>Urban wasteland</td>
</tr>
<tr>
<td>North</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riwaka, Nelson</td>
<td>41°4'202&quot; S, 172°59'194&quot; E</td>
<td>Roadside verge</td>
</tr>
<tr>
<td>Marshland, Canterbury</td>
<td>43°28'491&quot; S, 172°39'205&quot; E</td>
<td>Urban wasteland</td>
</tr>
<tr>
<td>Springston, Canterbury</td>
<td>43°39'684&quot; S, 172°25'136&quot; E</td>
<td>Roadside verge</td>
</tr>
<tr>
<td>Greymouth, Westland</td>
<td>42°26'809&quot; S, 171°12'355&quot; E</td>
<td>Riverbank</td>
</tr>
<tr>
<td>Makarewa, Southland</td>
<td>46°20'005&quot; S, 168°21'143&quot; E</td>
<td>Agricultural land</td>
</tr>
</tbody>
</table>

Starting in late spring (November), seedlings from each wild turnip population and rape plants were sown and grown to flowering in 5-litre pots of potting mix (80% composted bark, 20% sand WAP 5, with Osmocote Plus 15-4.8-10.8, 3-4 months release at 2 kg/m³ and Dolomite lime at 2 kg/m³). Plants were kept in an open-ended fiberglass shelter, and without artificial lighting, ventilation, or heating.

2.2.2 Hand pollinations

In both years (1999 and 2000) six plants from each of the six wild turnip populations were used along with six rape plants. Two individual flowers in the turnip raceme were opened while at the yellow bud stage, emasculated and pollinated with mature rape pollen from each of six rape plants, resulting in 12 pollinations on the lower raceme of each wild turnip plant. In the first year this was repeated on the upper raceme giving a total of 24 pollinations on each turnip plant. Immediately following pollination the flowers were capped with aluminium foil and left for one week, after which the cap was removed and the silique left to ripen for later harvest of seed.

2.2.3 Interspecific hybrid screening

Harvested seeds were surface sterilised by immersion in 1% sodium hypochlorite (plus one drop of Tween 20) for 10 min, followed by 3 rinses in sterile water. Seeds were sown onto the surface of nutrient medium consisting
of half strength MS salts (Murashige & Skoog 1962) at pH 5.8 solidified with 0.8% (w/v) Gibco bacteriological agar. This medium was autoclaved for 15 min at 103 kPa, then filter-sterilised chlorsulfuron was added to a final concentration of 10 μg/litre just before dispensing 50 ml into pre-sterilised 290-ml plastic pottles (80 mm base diameter x 60 mm high) (Carter Holt Harvey Plastic Products, Auckland, New Zealand). Seeds sown in each pottle were germinated at 24-26°C under light from cool white fluorescent lamps (80-100 μmol/ m²/sec, 16 h photoperiod). Hybrid seedlings were recognised by resistance to chlorsulfuron derived from the pollen parent. Only negligible root extension into the medium (<5 mm) occurs in herbicide-sensitive seedlings, whereas root extension may be up to 5 cm after 1 week for resistant seedlings (Conner et al. 1994). The viability of non-germinated seed was tested using the standard tetrazolium test (Hartmann & Kester 1983).

2.2.4 Flow cytometry

Eighty-six putative hybrids were recovered from the culture medium and sown in potting mix (as above). They were grown on to flowering and leaves sampled for flow cytometry. Two or three hybrids from each maternal plant were tested as described by Morgan et al. (1995). Briefly, nuclei were isolated after chopping leaves in Galbraith’s buffer, then treated with RNAase (DNAase-free) and stained with 50 μg/ml propidium iodide. Analysis was performed on an Epics Profile II flow cytometer (Coulter Electronics Inc.), fitted with an argon laser (488 nm) that operated at 15 mW. After calibration with barley (11.12 pg DNA/2C nucleus) and pea (9.73 pg DNA/2C nucleus), parsley (4.72 pg DNA/2C nucleus) was used as an internal standard with each sample.

2.2.5 Statistical analysis

The cumulative totals of pollination events resulting in siliques with and without seed for populations, years, and raceme position were compared using chi-square tests of independence. The effects of maternal wild turnip population, pollen donor, year, and raceme position on seed number and the percentage of dormant seed were analysed using ANOVA allowing for the hierarchical (nested) nature of the study design. The data were analysed separately for
each year (1999, 2000) and in an analysis which combined the lower raceme data for both years. Individual siliques were treated as replicates.

2.3 Results

2.3.1 Pollination success

Of the 1296 hand pollination events, 41% resulted in siliques with seed. The frequency of successful pollinations was affected by the population of wild turnip used as the maternal parent (Figure 2), and ranged from 31% (Greymouth) to 68% (Riwaka). There was a significant difference in the frequency of successful pollination on the upper and lower raceme of the wild turnip, with the lower raceme having almost twice as many successful pollinations than the upper raceme (Figure 3). When the results from the lower racemes of both years were compared there was no significant difference in the frequency of successful pollinations ($\chi^2 = 0.02; \text{d.f.} = 1; P = 0.96$).
Figure 2. Frequency of successful pollination after hand pollination of wild turnip maternal plants with rape pollen on the lower raceme over two years. Chi-square tests of independence established there were highly significant differences between the wild turnip maternal populations ($\chi^2 = 46.53; \text{d.f.} = 5; P < 0.001$).
Figure 3. Frequency of successful pollinations after hand pollination of wild turnip maternal plants with rape pollen in two raceme positions (year 1). Chi-square tests of independence established highly significant differences between the lower and upper raceme ($\chi^2 = 70.76$; d.f. $= 1$; $P < 0.001$).

### 2.3.2 Seed production

The average number of seeds developing following a successful pollination was 7.6 per silique. The majority (82.3%) of seeds germinated as chlorsulfuron-resistant progeny, indicating hybrid status (Table 3). The remainder comprised dormant seed (7%), fungal contaminated seed (10%), and those germinating as chlorsulfuron-sensitive progeny (0.7%). One hundred and seventy-two seeds germinated in the silique before normal seed maturity. Upon subsequent sowing, the majority of these pre-germinated seeds were lost to fungal contamination, with the remainder successfully recovered as chlorsulfuron-resistant progeny.
Table 3. Overview of the total number of seeds produced from hand pollination of wild turnip plants with rape pollen.

<table>
<thead>
<tr>
<th>Seed category</th>
<th>No. of seed</th>
<th>% of total seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated as chlorsulfuron-resistant progeny</td>
<td>3348</td>
<td>82.3</td>
</tr>
<tr>
<td>Dormant seed*</td>
<td>284</td>
<td>7.0</td>
</tr>
<tr>
<td>Contaminated seed†</td>
<td>408</td>
<td>10.0</td>
</tr>
<tr>
<td>Germinated as chlorsulfuron-sensitive progeny</td>
<td>30</td>
<td>0.7</td>
</tr>
<tr>
<td>Total seed†</td>
<td>4070</td>
<td>100</td>
</tr>
</tbody>
</table>

*Seeds that failed to germinate during the seedling screen. These were assumed to be dormant since from a sample of such seeds (n = 114), 96% were confirmed as retaining viability either by eventual germination or tetrazolium staining.
†Seed succumbing to fungal infections acquired in the silique.
‡Of these seeds, 172 (4.2%) pre-germinated in the silique before maturity. The majority of these viviparous seeds were lost to fungal contamination, with the remainder recovered as hybrids.

The wild turnip populations varied significantly in the number of seeds per silique following emasculation and pollination with rape pollen (Figure 4). The upper raceme produced less seed per silique than the lower raceme (Figure 5). No interactions were significant, and the year and the different rape pollen donors had no effect on the number of seeds produced in each silique (Figure 4 and Figure 5).
Figure 4. Mean seed number recovered from each silique (± standard error) after hand pollination of wild turnip maternal plants with rape pollen on the lower raceme over two years. ANOVA established there were significant differences between the wild turnip maternal populations ($F = 4.12; \text{d.f.} = 5, 60; P < 0.01$). There were no significant differences between years ($F = 0.01; \text{d.f.} = 1, 60; P = 0.918$) or the pollen parents ($F = 1.58; \text{d.f.} = 10, 60; P = 0.14$). The population x year interaction was not significant ($F = 1.7; \text{d.f.} = 5, 60; P = 0.138$).
Figure 5. Mean seed number recovered from each silique (± standard error) after hand pollination of wild turnip maternal plants with rape pollen in two raceme positions (year 1). ANOVA established there was a significant difference between the lower and upper raceme (F = 9.29; d.f. = 1, 572; P < 0.01), while the individual pollen parents did not differ significantly (F = 1.57; d.f. = 10,60; P = 0.14). No interactions were significant (0.065 < P < 0.530).

2.3.3 Verification of hybrid status

The DNA content in nuclei from a sample of the putative interspecific hybrid plants was analysed by flow cytometry to confirm their hybrid status. Flow cytometry results showed a clear differentiation between the two parent species, and between the species and the hybrids (Table 4). There was no overlap between these three groups, and no differences between the hybrids from different turnip populations, indicating the success of this rooting assay method for discerning between the species and the hybrids.
Table 4. Flow cytometry results from analysis of putative hybrids obtained from the hand pollination of maternal wild turnip plants with rape pollen.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>No. of plants tested</th>
<th>DNA content (pg/2C nucleus)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild turnip</td>
<td>7</td>
<td>1.18</td>
<td>1.14-1.19</td>
</tr>
<tr>
<td>Rape</td>
<td>4</td>
<td>2.56</td>
<td>2.53-2.58</td>
</tr>
<tr>
<td>Greymouth hybrids</td>
<td>7</td>
<td>1.88</td>
<td>1.80-2.01</td>
</tr>
<tr>
<td>Marshland hybrids</td>
<td>20</td>
<td>1.86</td>
<td>1.83-1.89</td>
</tr>
<tr>
<td>Massey hybrids†</td>
<td>14</td>
<td>1.89</td>
<td>1.85-1.92</td>
</tr>
<tr>
<td>Riwaka hybrids</td>
<td>17</td>
<td>1.90</td>
<td>1.86-1.97</td>
</tr>
<tr>
<td>Springston hybrids</td>
<td>11</td>
<td>1.89</td>
<td>1.83-1.97</td>
</tr>
<tr>
<td>Makarewa hybrids</td>
<td>16</td>
<td>1.88</td>
<td>1.83-1.90</td>
</tr>
</tbody>
</table>

† One additional plant from the Massey population was confirmed as having the diploid genome size of wild turnip (1.19 pg DNA/2C nucleus). This was assumed to be a mislabeled plant.

2.3.4 Analysis of dormancy results

Analysis of the frequency of dormant seeds showed that wild turnip populations and the different rape pollen donors did not effect the level of dormancy in the resulting seeds in either year. However, the upper and lower racemes exhibited a highly significant difference in the frequency of dormant seed recovered, with the upper raceme producing 6-fold more dormant seed (Figure 6).
2.3 Discussion

Herbicide-resistant genes are useful tools for studying the introgression of genes from one species to another. When resistance is conferred by a single locus, homozygous for dominant alleles, it offers an excellent genetic marker to monitor interspecific hybridisation (Conner & Field 1995). Previous studies on interspecific hybridisation between *Brassica* species have relied on morphological traits (Palmer 1962; Landbo *et al.* 1996) or molecular markers (Jørgensen *et al.* 1995; Wilkinson *et al.* 2000) to identify hybrids. In this study a simple and inexpensive method for screening large progeny populations for herbicide-resistant hybrids was used, and the results confirmed using flow
cytometry to verify the accuracy of the seedling screening method. This experimental approach provides a convenient basis for risk assessment of transgenic brassica plants.

The frequency of successful pollinations was influenced by both the maternal wild turnip population (Figure 2) and the position of the pollination event on the raceme (Figure 3). Landbo et al. (1996) speculated that wild turnip "plants and populations may differ in their ability to select against B. napus pollen on the stigma", suggesting wild turnip populations may differ in their ability to form interspecific hybrids. The results of this study confirm the existence of disparity in the frequency of hybridisation between rape and different wild turnip populations.

As expected, over 99% of the successfully germinated seeds were recovered as putative hybrid progeny following hand pollination of wild turnip with rape pollen (Table 3). From the large number of progeny recovered with herbicide resistance, 85 putative hybrid plants were verified by flow cytometry as having the expected triploid status (Table 4). This validates the reliability of the herbicide resistance assay for identifying the interspecific hybrids. The low frequency non-hybrid progeny (0.7%) may have germinated from matromorphic seed. In the Brassicaceae, pollination of stigmas with foreign pollen has been shown to stimulate the development of the female gamete, either as an unreduced gamete or as a doubled-haploid, resulting in a seed without any chromosomes contributed by the pollen parent (Scheffler & Dale 1994). In the present study, such matromorphic plants are expected to have the genome content of the maternal wild turnip parent. The nature of the screening method for herbicide resistance prevented the recovery of viable seedlings from those plants, so confirmation of their origin via flow cytometry was not possible.

Vivipary (precocious germination) has been previously recorded in turnip × rape crosses when turnips were used as the maternal plant (Calder 1937; Hauser & Østergård 1999; Farnsworth 2000). This biological phenomenon was confirmed in the present study, with 4.2% of the total seeds being viviparous (Table 3). All the seedling progeny recovered from these viviparous seeds were hybrids. However, the vulnerability of viviparous seed to fungal infections, desiccation, and an inability to disperse is considered to result in an important loss of hybrid
seeds (Hauser & Østergård 1999) and therefore the potential for interspecific gene introgression.

Seed number per silique following interspecific hybridisation varied among the maternal populations (Figure 4). The higher seed output of some populations may reflect a differential adaptation to the artificial environment, but is also indicative of the potential for differences between plant populations. Higher seed production is considered to improve the fitness of a plant, and improve the opportunities for gene introgression (Hauser et al. 1998a).

Differences between the upper and lower raceme in the production of interspecific hybrids have not been previously reported. However, the frequency of outcrossing in rape varied between 39% at the bottom of the raceme and 11% at the top of the raceme which was attributed to environmental influences (Becker et al. 1992). The present study found the frequency of interspecific pollination success on the lower racemes was almost twice that of the upper racemes (Figure 3). The number of seeds per silique was also significantly higher on the lower raceme compared with the upper raceme (Figure 5), whereas the frequency of seed dormancy was 6-fold higher on the upper raceme (Figure 6). The change in seed number is physiologically based since the siliques at the top of the plant are usually smaller than those produced in the early stages of flowering (Clarke 1979).

The interspecific hybrid seed between rape and wild turnip has been previously reported as not exhibiting any primary dormancy (Landbo & Jørgensen 1997; Linder 1998). The present study found an appreciable frequency of primary seed dormancy for the interspecific hybrids (7%) (Table 3). In addition, the frequency of dormancy was markedly higher in the upper raceme (Figure 6), a finding which has not previously been reported. This has potential management implications for the commercial release of transgenic rape cultivars. If seed is not allowed to form on the upper raceme of wild turnip plants, then the opportunities for interspecific hybrids to enter the weed seed bank as dormant seed is substantially reduced. Non-dormant hybrid seed will germinate soon after ripening and falling from maternal plants during late summer/autumn in New Zealand, whereupon they may die as a result of the winter climate, or be
controlled by cultural means. Mowing the margins of rape fields and roadsides to prevent the upper raceme of wild turnip from flowering may decrease long-term weed problems from dormant hybrid seed, and reduce perceived concerns from releasing transgenic rape.

The frequency of pollen-mediated gene introgression from rape to wild turnip depends on the degree of sexual compatibility between the two species. Hand pollination provides a basis for assessing the potential frequency of hybrid progeny in different environments such as the field, and under open pollination in the glasshouse. This study has established there is a high level of compatibility between the rape line used in these experiments and wild turnip populations collected from a wide range of New Zealand habitats and locations.
Chapter 3: The incidence of interspecific hybrids resulting from open field pollination of wild turnip populations with herbicide-resistant rape pollen

3.1 Introduction

The introduction of transgenic technology to agricultural production systems has been politically controversial (Nap et al., 2003). The environmental release of transgenic crops is often considered to pose ecological risks (Conner et al., 2003). One of the major issues of concern involves the introgression of transgenes to related species (Ellstrand, 2001) and the potential for these genes to increase weedy attributes (Conner et al., 2003). Rape and wild turnip is a crop-weed combination that presents a potential risk for the escape of transgenes (Scheffler & Dale, 1994, Bourdôt et al., 1999). Interspecific hybridisation between rape and turnip (both wild and cultivated) is not a new issue and has been recognised for many years, with Darwin (1876, p. 393) citing an example of “A writer in the ‘Gardeners’ Chronicle’ (1855, p.730) says that he planted a bed of turnips (Brassica rapa) and of rape (B. napus) close together, and sowed the seed of the former. The result was that scarcely one seedling was true to its kind, and several closely resembled rape.” Even so, the interspecific hybridisation of rape and wild turnip and subsequent gene introgression has not presented a major agricultural problem in the past, except for the production of genetically pure seed (Manasse, 1992).

Rape plants are self-compatible and will readily self-pollinate, though they are recorded as having 12-47% intraspecific outcrossing in field situations (Becker, Damgaard, & Karlsson, 1992). Becker et al. (1992) found an increase in outcrossing from the bottom to the top of plants, while Leckie et al. (1993) found no effect of flower position on outcrossing. The capacity for rape and wild turnip to hybridise has been well established from hand pollination studies (e.g.(Sutton, 1908; Kajanus, 1917; U, 1935; Beversdorf, Weiss-Lerman, Erickson, & Souza Machado, 1980; Bing, Downey, & Rakow, 1991).
The frequencies of natural hybridisation between rape and wild turnip have been reported in controlled field experiments from different regions of the globe, and have produced widely differing results. Isolated turnip plants, being self-incompatible (Palmer, 1962; Holm et al., 1997) would be expected to have a high frequency of hybrid progeny if isolated from other turnip plants and planted in a rape field. In a previous New Zealand study, interspecific hybrids were found to comprise between 10 and 88% of the progeny when rape and wild turnip were grown together in densities ranging from 1:1 in an isolated field, to single rape/turnip plants isolated in at least 10 m² of the other species planted as a crop (Palmer, 1962). In Canada hybrids were found to comprise less than 1% of the progeny from wild turnip plants when interplanted with rape plants at an unspecified ratio (Bing et al., 1991). Isolated B. campestris plants in rape fields are recorded as having a hybrid seed frequency of 93% (Jørgensen et al. 1996).

An extensive study of natural wild turnip populations growing sympatrically with rape in Britain reported one plant out of 505 new recruits to the wild populations was a hybrid, although the ratio of rape:turnip plants was not stated (Wilkinson et al., 2000). A more recent study in the USA found there were differences between oilseed rape lines for interspecific hybridisation frequency (0.7%-16.9%) and variation between the lines ability to hybridise in controlled conditions and in the field (Halfhill et al., 2002). A further study of the brassica species occurring in Canterbury, New Zealand showed that natural interspecific hybrids exist in the ruderal environment at a very low frequency (Heenan et al., 2004).

The variability of data in these reports and the inconsistent experimental designs has resulted in the natural rate of introgression between rape and wild turnip being a controversial issue (Rieger et al., 1999). The relative importance of differences between populations/genotypes, environments/seasons, or ratio of the parent species has not been resolved when considering the differences between these reports. Resolving these issues is important for evaluating the ecological impact of releasing transgenic brassica crops.
As part of a risk assessment study for the release of transgenic rape plants in New Zealand, this chapter investigates the naturally occurring frequency of interspecific hybrids in the field between herbicide-resistant rape (*Brassica napus*) and wild turnip (*Brassica rapa* DC. var *oleifera*) populations from a range of habitats in New Zealand. Experiments were conducted over 4 years using six wild turnip populations and with ratios of 400:1 and 1:1 wild turnip to rape plants.

### 3.2 Materials and methods

#### 3.2.1 Plant materials

Wild turnip populations from a large range throughout New Zealand were sampled as described in Chapter 2. Five populations (Massey, Riwaka, Marshland, Springston and Makarewa) as described in Table 2 and with the addition of Richmond (Richmond, Nelson 41°21'133"S, 173°10'361"E, roadside verge) were tested in the field for frequency of interspecific hybridisation with rape. The non-transgenic herbicide-resistant rape plants, homozygous at a single locus for a dominant mutation conferring resistance to chlorsulfuron, were from the same population as that used in Chapter 2.

#### 3.2.2 Field trial design

The experiment was conducted over the summers of 1997/98, 1998/99, 2000/01 and 2001/02, subsequently referred to as years 1, 2, 3 and 4. Six wild turnip populations as described above were planted in the field each spring as 2x3m plots with approximately 800 seeds per plot. Rape plants were sequentially sown in four litre pots and grown in the glasshouse to ensure synchrony of flowering. When wild turnip flowering commenced in the field, two flowering rape plants per plot were introduced to the field until wild turnip flowering was completed.

In year 2 plants were also established in a 1:1 ratio of rape: turnip. The seeds were sown in the glasshouse, and the plants transferred to the field at the three true-leaf stage. The 1:1 ratio was achieved by planting 6 rows, with one turnip population in each row. Within the row, turnip plants alternated with rape
plants, and the plants were approximately 0.2m apart. Rows were approximately 0.5 m apart. When fully grown, the flowering plants were touching, both within and between the rows.

Over the five year period experiments were conducted in an area of 1 km radius at the Lincoln University Iverson Field Research Unit, with the sites never closer than 500m to that of the previous years. Plants were left to pollinate naturally, and the seeds from ten randomly selected wild turnip plants were individually harvested at maturity.

Seeds were cleaned, then counted twice with a Numigral II seed counter, and the mean of the two counts used in the analysis. In the first year 200 seed aliquots per plant were screened for interspecific hybrids, while in subsequent years the entire seed harvest was screened.

3.2.3 Seed screening

Germinated seedlings were screened for chlorsulfuron resistance as a marker for the presence of interspecific hybrids as described in Chapter 2.

3.2.4 Statistical analysis

Results were analysed using ANOVA to explore year and population effects on seed number per plant and frequency of interspecific hybrids recovered. The individual wild turnip plants were treated as replicates.

3.3 Results

Over the four years of field experimentation 474,879 seeds were harvested from the six wild turnip populations, of which 263,454 seeds were screened for interspecific hybrids. Due to limitations imposed by seed availability, it was not possible to use all wild turnip populations every year.

The influence of wild turnip populations on interspecific hybridisation with rape was investigated by statistical comparison among the four–six populations used each year. In all four years no significant population effect on the frequency of interspecific hybrids was observed (Figure 7). Furthermore, no significant differences were observed between the wild turnip populations in seed
production per wild turnip plant in years 1, 3, and 4, although in year 2 populations varied significantly and ranged from 1000 – 3000 seeds per plant (Figure 8).
Figure 7. The frequency of interspecific hybrids recovered from wild turnip populations grown in the field with rape. ANOVA established that there were no significant differences between populations in Year 1 ($F=0.97, df=3, 36, P=0.416$), Year 2 ($F=1.24, df=5, 54, P=0.305$), Year 3 ($F=0.19, df=4, 73, P=0.943$) or Year 4 ($F=0.87, df=5, 114, P=0.505$). [Note: a different scale is used on the abscissa in Year 1.] Error bars represent standard errors.
Figure 8. Seed production of wild turnip populations. ANOVA established no significant difference between populations in year 1 ($F=0.52$, $df=3$, 36, $P=0.671$), year 3 ($F=0.50$, $df=4$, 73, $P=0.737$) and year 4 ($F=1.16$, $df=5$, 114, $P=0.335$). Significant differences between populations were apparent in year 2 ($F=3.19$, $df=5$, 54, $P=0.014$). [Note: a different scale is used on the abscissa in Year 1.] Error bars represent standard errors.
For the three populations (Marshland, Springston and Makarewa) used in all four years, no population effect or population x year interaction was observed for the frequency of interspecific hybrids occurring in the field (Figure 9). However, a highly significant year effect was observed, with Year 1 having a significantly higher frequency of wild turnip x rape hybrids than years 2, 3 and 4. The seed produced per wild turnip plant showed no population effect or populations x year interaction, but again a highly significant year effect was observed (Figure 10). Substantially more seeds per plant (3-10x for individual plants) was produced in year 1 compared to years 2, 3 and 4.

Figure 9. The frequency of interspecific hybrids recovered from three wild turnip populations grown in the field with rape over four years. ANOVA established that there were highly significant differences between the years (F=7.42, df=3,155, P<0.001). The wild turnip populations were not significantly different from one another (F=0.75, df=2,155, P=0.473) and the interaction between years and populations was not significant (F=0.84, df=6,155, P=0.541). Data is pooled across populations for each year. Error bars represent standard errors.
Figure 10. Seed production of wild turnip over four years. ANOVA established that there differences between the years (F=38.98, df=3,155, P<0.001). The wild turnip population did not significantly effect seed number (F=0.51, df=2,155, P=0.603) and the population x year interaction was not significant (F=0.58, df=6,155, P=0.748). Data is pooled across populations for each year. Error bars represent standard errors.

A higher ratio of rape to wild turnip plants produced a significantly higher frequency of interspecific hybrid progeny, with no significant differences observed between populations (Figure 11). The seed production per wild turnip plant was also higher under the higher ratio of the two parent species, also with no significant differences in seed production between the wild turnip populations (Figure 12).
Figure 11. Percent of interspecific hybrid progeny recovered from wild turnip maternal plants hybridising with rape in the field at two different ratios of wild turnip and rape. ANOVA established there were highly significant differences between the two ratios ($F=10.95$, $df=1, 89$, $P=0.001$). Populations were not significantly different ($F=0.97$, $df=4, 89$, $P=0.427$). The interaction between populations and ratios was not significant ($F=0.85$, $df=4, 89$, $P=0.500$).

Figure 12. Seed numbers recovered from wild turnip maternal plants established with two different ratios of rape to wild turnip. ANOVA established there were significant differences between the two ratios ($F=67.61$, $df=1, 90$, $P<0.001$). Populations were not significantly different ($F=0.99$, $df=4, 90$, $P=0.417$). The interaction was not significant ($F=0.91$, $df=4, 90$, $P=0.464$).
Over this four year period of experimentation the incidence of interspecific hybridisation between wild turnip and rape was consistently low (<0.1%-2.1% total seed on wild turnip plants). However a large variation in mean seed production on the wild turnip populations was also apparent (416-6972 per plant). A calculation of the absolute number of interspecific hybrids on a per plant basis (total seed per plant x % interspecific hybrids) over each population each year reveals a wide range of interspecific hybrid production. With approximately 400 wild turnip plants for every rape plant the number of interspecific hybrids developing on each wild turnip plant ranged from a mean of 0.146 to 0.4 interspecific hybrids per wild turnip plant. With a 1:1 ratio of these two parent species a range of zero to 161 interspecific hybrids per wild turnip plant was observed.

3.4 Discussion

The risk analysis for the field release of genetically engineered herbicide-resistant crops requires experimental data on the introgression of new genes into closely related species in the natural environment. This experiment used a non-transgenic mutant rape to provide a model of possible gene flow to wild turnip populations originating from a wide geographical spread of habitats in New Zealand. The experimental design chosen mimics rare escapes of rape seed from cultivation into wild turnip populations established in ruderal environments, predominantly roadsides. The small plot size contained more individuals than most observed ruderal wild turnip populations (Heenan et al., 2004). The small area ensured that all turnip plants were potentially exposed to rape pollen by being within 3 m of the rape plant, where 50% of pollen produced is deposited (Lavigne et al., 1998).

The wild turnip populations used as maternal plants had no effect on the frequency of interspecific hybridisation with rape (Figure 7 and Figure 9). This is in contrast to the results obtained in Chapter 2. It may be that in this field study the hybridisation frequencies are so low the population differences could not be ascertained given the sample sizes used. High variation in the frequency of interspecific hybridisation (0-93%) among seed collected from wild turnip populations growing in a range of habitats in previous studies was attributed to
population effects (Jørgensen and Andersen 1994, Landbo et al., 1996), a situation not observed in this study (Figure 7 and Figure 9). The frequency of hybrid progeny was low (<0.1 – 2.1%) (Figure 7 and Figure 9) compared with a previous study of New Zealand populations of cultivated turnip and wild turnip hybridised to crops of swede and rape (Palmer, 1962). In this previous study, rates of interspecific hybridisation ranged from 3.9%-94.6% with the highest frequencies of interspecific hybridisation obtained where turnips were isolated in rape fields. The wild and cultivated turnips used in these previous experiments were not distinguished in the presented results (Palmer, 1962). Studies have shown that ‘weedy’ B. rapa in the New World, which includes New Zealand, are likely to be escapes from cultivation, and to have similar genetic characteristics to cultivated turnips (Crouch et al., 1995). Differences are therefore unlikely to have occurred between wild and cultivated turnips in the Palmer (1962) study. Bing et al., (1991) also examined the crossing of rape and wild turnip in the field, and found frequency of hybridisation to be 0.99% when wild turnip was the maternal plant.

The frequency of hybridisation reported here is more analogous to those presented which used wild turnip as the female plant. It was intended by the experimental design to imitate naturally occurring sympatric populations. In south-east England, 15 000km² was surveyed to find sympatric populations of rape and wild turnip, and every newly recruited plant in the subsequent year was screened for hybrid status (Wilkinson et al., 2000). Frequency of hybridisation was measured at 1/505 plants screened, or 0.20% (Wilkinson et al., 2000), and at 0.4%-1.5% in a smaller but similar experiment in Berkshire and Oxfordshire (Scott & Wilkinson, 1998). A subsequent paper by Wilkinson et al. (2003) recorded a range of hybridisation rates (0-17.5%) in different locations throughout mainland Britain. One putative B. napus x B. rapa hybrid was reported in a substantial survey of Brassica in Canterbury, New Zealand (Heenan et al., 2004). Although no frequency information is given in Heenan et al. (2004), the small number of hybrids found in the survey of a large area indicates a low (but possibly important) frequency of hybridisation.

The frequency of hybridisation found in this experiment is within the range of the English and Canadian experiments, but lower than the mean of the Danish and
previous New Zealand (Palmer, 1962) experiments. In all these experiments the highest frequency of hybridisation occurred when isolated self incompatible turnip plants were grown in fields of rape (eg Palmer, 1962; Jørgensen et al., 1996). It was recorded that a 1:1 mixture of *B. campestris* and rape plants had a 13% hybrid seed frequency (Jørgensen et al. 1996). The data presented here confirms this pollen dose effect, but the overall frequency of interspecific hybridisation at both ratios is lower than that reported in the previous studies (Figure 11).

Seed production was highly significantly affected by season, with year 1 producing 3.3 times more seed than the next most productive season – indicating it was a good year for wild turnips (Figure 8). Year 1 also produced the greatest frequency of interspecific hybridisation (Figure 9). In year 2 there was a population effect on seed production (Figure 10), but this was not observed in the other years. Hand pollination resulted in differences in the production of interspecific hybrid seeds between wild turnip populations (Chapter Two). This population effect on seed production was not apparent in the field (Figure 10), although as virtually all seed produced in the field resulted from intraspecific pollination, the effect may have been obscured.

The experiment investigating the influence of the relative numbers of sympatric wild turnip and rape plants suggested a substantially higher seed production when the parent species were growing at a 1:1 ratio (Figure 12). However, this is probably an artifact of the experimental design, as plant spacing was confounded with the ratio of the parent species. When established at a 1:1 ratio hand planting resulted in each plant having an available space of 0.09 m², compared to only 0.0075 m² when plants were sown at the 400:1 ratio. The relationship between plant spacing has been examined in many crops (eg (Mead, 1966), and there is a consistent correlation of greater individual plant productivity with increased space.

Seed production is an important factor that has not always been considered in gene flow studies. Seed production in these experiments ranged from a mean of 416-6972 per plant with individual plants within treatments varying from 0-20,310 seeds produced (data not presented). Individual wild turnips apparently
vary in their fecundity, a typical characteristic of highly heterozygous weed populations (Jasieniuk & Maxwell, 2001). A lower frequency of hybridisation combined with a high number of seed produced will result in a larger number of interspecific hybrid seeds relative to the same frequency combined with a low seed production. A greater number of interspecific hybrids are likely to increase the chances of hybrid survival in the subsequent seasons. The mean absolute number of hybrids per wild turnip plant in this study ranged from 0-0.4. When this is coupled with the extremely wide range in seed production of the wild turnip plants, it is possible that total seed production per plant could be a more important factor than the frequency of hybridisation in influencing the number of interspecific hybrids produced per plant.

Whether the addition of these rare interspecific hybrid seeds to the weed seed bank will contribute to gene introgression between the species is dependent upon the fitness and fecundity of the resulting interspecific hybrid seedlings in the field.
Chapter 4: Relative importance of honeybees and wind as vectors for pollen-mediated gene flow from rape to wild turnip

4.1 Introduction

The release of transgenic crops has raised concerns about possible gene flow to surrounding crops and weed populations (Eastham & Sweet, 2002; Conner et al., 2003). Genes are most likely to introgress from transgenic crops to other plant populations via pollen. This requires a vector to effect pollen dispersal. The crop and weed combination that presents one of the greatest risks of gene flow involves rape (Brassica napus L.) and wild turnip (Brassica râpa subsp. sylvestris (L.) Janchen). A few studies have reported natural hybridisation of wild turnip and rape (e.g. Palmer, 1962; Bing et al., 1991; Jørgensen & Anderson, 1994; Scott & Wilkinson, 1998). They have shown wide variation in the frequency of production of hybrid progeny, from 0-95%. There are no reports on the natural vectors to effect pollen transfer for such interspecific hybridisation, and these are generally assumed to be the same those in common for the parent species, namely wind and insects.

The rape flower is formed from four petals, six stamens and two fused carpels. The stamens are further divided into two groups, two short stamens with introrse anthers, and four long stamens. It has four nectaries, two of which are concealed between the inner side of the bases of the short stamens which contain lower concentrations of sugars and may attract different insect species (Eisikowitch, 1981). The flower is initially configured for insect pollination with the long stamens extrorse and higher than the stigma (Williams, 1984), but as the stigma elongates it positions closer to the anthers which simultaneously curve towards the flower center resulting in the pollen covering the top and side of the stigmata (Eisikowitch, 1981), with the higher position of the stigma enhancing the likelihood of wind pollination (Williams, 1984). Flower opening can occur at any time of the day, which maximises the opportunities for pollination.
Pollination of rape is known to occur by three different mechanisms: wind, bees and mechanical means (Timmons et al., 1995). Rape has large sticky pollen grains typical of entomophilous species, which are considered not to be transferred by wind alone (Eisikowitch, 1981). Physical movement of the stamen (flicking) is required to release pollen from the anther, which can be effected by mechanical movement of the plant by wind or by insects (Eisikowitch, 1981). Such mechanical release of pollen must be easily achieved since dense clouds of pollen (7.5 to 5295 g m\(^{-3}\)) have been reported to form over rape crops (Williams, 1984), which may be important for gene flow from rape crops. Up to 22 pollen grains m\(^{-3}\) at a distance of 1.5 km from the rape pollen source have been recorded (Timmons et al., 1995), suggesting that wind-mediated pollen dispersal may occur over long distances. Although wild turnips are considered to be predominantly insect pollinated (Holm et al., 1997), wind and mechanical mechanisms are likely to play a similar role as for rape.

Several studies have investigated pollination of rape in efforts to improve the seed yield of the crop (e.g. Free & Nuttall 1968; Williams, 1978; Mesquida et al., 1988; Free, 1993). Providing insect vectors in rape fields has resulted in higher seed production (Free & Nuttall, 1968). In a field where bees are available the rape plants have about 30% outcrossing, with the later flowers less inclined to outcross (Becker et al., 1992). Bees are known to forage in a radius of two km around their hive (Ramsay et al., 1999) and therefore have the potential to move pollen over a distance of four km. Bees become covered in the sticky rape pollen upon visiting flowers, and the pollen can remain on the bees for several foraging trips to and from the hive (Ramsay et al., 1999). Bees are known to often remain loyal to one plant species on each foraging trip (Zahavi et al., 1983; Proctor et al., 1996).

Knowledge of both the vectors that effect pollination events for interspecific hybridisation and the nature of any barriers that minimise interspecific hybridisation are important for managing the incidence of gene flow between crops and other species, including transgenic crops. This study investigated the relative importance of entomophily and anemophily as pollination mechanisms to effect interspecific hybridisation and pollen-mediated gene flow from rape to wild turnip. These two species were enclosed with either bees or a wind-source
to effect pollination and progeny for the wild turnip screened for a herbicide-resistant marker gene from the rape parent. To help explain results, flowers of rape and wild turnip were photographed for UV emittance spectra to investigate whether insects could visually discriminate between the two species.

4.2 Materials and methods

4.2.1 Plant material

A rape line (30a), homozygous at a single locus for a dominant mutation conferring resistance to chlorsulfuron (Conner et al., 1994), was used along with five populations of wild turnip collected from diverse habitats over the southern 950 km of New Zealand (Massey, Riwaka, Marshland, Greymouth, Makarewa (Table 2)). Plant pots used were 8 litres in volume. Flowering was synchronous.

4.2.2 Experimental design

Two 5m x 2.5m x 1.7m high glasshouses were used at the Nursery and Greenhouse Center of Lincoln University. Twenty plants from each of five wild turnip population were used with ten plants being randomly allocated to each glasshouse. These plants were further divided into two groups of five, and placed on either the right or left side of the glasshouse. The two sides of the glasshouse acted as blocks for a complete randomized block design of populations. Each unit within a block contained five turnip plants and five rape plants in an alternating pattern. The ten plants were arranged in two rows, with each row consisting of alternating rape and wild turnip plants, resulting in one of the rows having three wild turnip plants and two rape plants, and the other row having two wild turnip plants and three rape plants. Row patterns were alternated resulting in a checkerboard pattern of the two species. Plants within each block were 20 cm apart, while there was a 30 cm gap between blocks. A total of one hundred rape plants were required for both glasshouses. The trial was repeated over two years, in the autumns of 1999 and 2000.

When flowering commenced, a small honey bee (Apis mellifera L.) hive consisting of one brood super was placed in one glasshouse. A large stationary
fan was placed in the other glasshouse at the height of the flowers. The volume of air moved by this fan was 0.57 m$^3$ s$^{-1}$, as measured with a Pitot tube anemometer, at a speed of 4.15 m s$^{-1}$ as determined with a hand held anemometer. The position of the fan in the greenhouse was changed three times per day at 0800, 1200 and 1600 hours to ensure an even wind coverage over the plants and ran 24 h per day. The flower racemes were carefully tied to stakes every two days in an attempt to minimise raceme movement by the wind and consequent mechanical pollination. After two weeks the next four flower buds on each raceme of every wild turnip plant due to open were removed from each raceme and this position tagged. The fan and the bees were then exchanged between the two glasshouses. After a total of four of weeks pollination period, both the bees and the fan were removed, the raceme marked at the position of the last flower pollinated and the plants left to mature their seed. The two treatments on each plant were harvested separately.

4.2.3 Screening for interspecific hybrids

As described in Chapter 2.

4.2.4 Statistical analysis

The effects of maternal wild turnip population, pollination vector, side of glasshouse and year on seed number and hybrid frequency were analysed using ANOVA allowing for the hierarchical (nested) nature of the study design. Individual siliques were treated as replicates.

4.2.5 Photography of flower UV reflectance

Rape and turnip flowers were photographed for their light reflectance patterns under both visible and UV light essentially following the methods of Primack (1982). UV reflectance was photographed using an electronic flash light (Hensel Minispot 250) with Ilford 828 UV filter (Wratten 18A) onto Kodak T-Max 400 B&W film.
4.3 Results

Bees were observed foraging throughout the day on both rape and wild turnip plants. There was a mean seed yield of 1715 seeds per half plant (each plant received two treatments). Bee pollination resulted in the production of approximately twice as many seeds on wild turnip plants as wind pollination (Figure 13). Differences in seed production were also apparent for wild turnip populations, the two glasshouses and the two years. The glasshouse effect was most likely a consequence of differing light levels resulting from different screens over the vents, and the year effect from a minor outbreak of blackleg fungal disease in one year. The overall high seed production from both pollination vectors provides a valid basis from which to assess the frequency of interspecific hybridisation between wild turnip and rape resulting from bee versus wind pollination. Interspecific hybridisation frequencies resulting from the transfer of rape pollen to wild turnip plants were low, varying between five and seven percent across the populations. There were no statistically significant differences among populations in the frequency of hybrids recovered (Figure 14). Since there were also no significant effects on frequency of hybrids recovered from glasshouses or years (Figure 14), results were pooled to give greater sensitivity in testing the significance of differences between the two pollination vectors. This established that wind pollination resulted in a highly significant increase in the frequency of interspecific hybrids compared to bee pollination (Figure 15).
Figure 13. Mean number of seeds recovered from each wild turnip plant pollinated with either wind or bees. ANOVA established significant differences between wind and bees ($F = 52.46; df = 1,144; P < 0.001$). Error bars represent standard errors.
Figure 14. Frequency of interspecific hybrid progeny recovered on wild turnip plants after wind or bee pollination in glasshouses with rape and wild turnip plants. ANOVA established that there were no significant differences between wild turnip populations (F = 0.56; df = 4,81; P = 0.69), glasshouses (F = 0.56; df = 3,81; P = 0.64) or years (F = 2.86; df = 1,81; P = 0.09).
Figure 15. Frequency of interspecific hybrid progeny recovered on wild turnip plants after either wind or bee pollination in separate glasshouses with rape and wild turnip plants. ANOVA established that there are significant differences in the two pollination methods (F = 8.71; df = 1,269; P = 0.01). Error bars represent standard errors.
Although wind pollination produced a higher frequency of interspecific hybrids between wild turnip and rape (Figure 15), the total seed production on wild turnip plants under wind pollination was substantially less (Figure 13). Therefore the absolute number of interspecific hybrids produced on each wild turnip plant may further differ between bee and wind pollination. Calculations from the pooled data over all experiments results in an average of 2.5 and 3.6 interspecific hybrids produced over two weeks of seed set on wild turnip plants for wind and bee pollination respectively.

To further investigate the overall low frequencies of interspecific hybridisation resulting from bee pollination, the light reflectance patterns from flowers of rape and wild turnip were examined. When photographed under visible light, the rape flowers had a similar appearance to all five wild turnip populations (Figure 16).
16). In contrast, when only the UV reflectance is considered the rape flowers are very light in appearance and markedly different from the very dark appearance of all five wild turnip populations (Figure 16).

4.4 Discussion

Artificial pollination establishes the potential for interspecific hybridisation to occur between two species, while open pollination in the field is an indicator of how often effective interspecific pollination occurs under natural conditions. Large discrepancies between the frequency of interspecific hybrids obtained by hand and by field pollination are often apparent. This is well illustrated by wild turnip and rape, with very high frequencies of interspecific hybrids arising from artificial pollination and comparatively low frequencies from open field pollination (e.g. Bing et al., 1991; Jenkins et al., 2001; Chapters 2 and 3). By examining the relative effectiveness of pollination vectors to hybridise two species, the differences in the frequency of hybridisation achieved by hand and field pollinations may be reconciled, and management protocols for minimising interspecific hybridisation may be determined.

Although three pollination mechanisms (wind, bees and mechanical) could be responsible for interspecific hybridisation between wild turnip and rape in the field, mechanical pollination was deliberately not included in the experimental design. Mechanical pollination requires physical contact between flowers of the two species which should not occur together in a well managed rape field. Wild turnips are well known to contain antinutritional factors that contribute to poor oil and forage quality to the rape crop (Appelqvist & Ohlson, 1972). Throughout the experiments in this study individual plants were tied to prevent flowers of adjacent plants from physical contact in order to minimise mechanical pollination, although some pollen movement may still have been facilitated during plant maintenance.

The honey bee is not the only insect pollinator that forages on rape flowers, but it is the most important. Other effective pollinators include Bombus spp. and hoverflies (Langridge & Goodman, 1982). Oilseed rape has a high concentration of sugars in the nectaries at the base of the four inner stamens
(Free & Nuttall, 1968) and bees will travel up to three kilometres to collect nectar (Ramsay et al., 1999). The entomophilic nature of rape pollen was further confirmed by Eisikowitch (1981), who demonstrated the inability of the pollen to be released from anthers using a hairdryer which blew air at 6 m s$^{-1}$ unless the anther was also 'flicked'. The enclosed controlled experiments in the present study clearly establish that bees alone are considerably more effective than wind alone for the pollination of wild turnip flowers. Over all five wild turnip populations, bee pollination resulted in a doubling of seed set relative to wind pollination (Figure 13).

Although seed set resulting from bee and wind pollination differed in wild turnip, both vectors effected high seed production, which provided a valid basis to assess the relative importance bee and wind pollination for interspecific hybridisation between wild turnip and rape. Overall the frequency of interspecific hybrids was low with the highest frequency recorded at 7.2% (Figure 14). There were no differences between the wild turnip populations in the frequency of interspecific hybridisation from rape pollen (Figure 14).

Wind was more effective than bees as an effective interspecies pollen vector (Fig. 3), but this observation must be tempered with the result of lower seed production from wind pollination (Figure 13). The absolute number of interspecific hybrids produced on each wild turnip plant was similar for both wind and bee pollination. Wild turnip is a self-incompatible species (Holm et al., 1997), though cultivated forms do set some seed when self-pollinated by hand (Williams, 1978). The wild turnip populations used in these experiments have consistently failed to set selfed seed, thereby confirming self-incompatibility. Therefore in these experiments all seeds recovered from the wild turnip plants must have been derived from pollen from another plant. Although no accurate quantification of the number of flowers was made for each plant, general observations indicated that plants of each species produced a similar number of flowers. Since rape anthers produce twice as many pollen grains as wild turnip anthers (Hauser et al., 1997), and there were an equal number of wild turnip and rape plants in each treatment, the expected frequency of interspecific hybrid progeny on the wild turnip plants is well over 50% if there are no hybridisation barriers.
Given that artificial pollination is highly effective at recovering interspecific hybrids between wild turnip and rape (e.g. Bing et al., 1991; Jenkins et al., 2001), the low frequencies of interspecific hybridisation observed in this study clearly demonstrates that effective barriers to hybridisation exist in nature. A key hybridisation barrier that may account for the low frequency of interspecific hybrids may involve pollen competition. Hauser et al. (1997) found there was preferential exclusion of interspecific hybrids when mixed pollinations of rape and wild turnip pollen were placed on the turnip stigma. This may account for the overall low levels of interspecific hybridisation resulting from both bee and wind pollination (Figure 15), especially given the high potential for interspecific hybridisation in these experiments.

The substantially lower frequency of interspecific hybrids resulting from bee pollination relative to wind pollination (Figure 15) suggests that bees can discriminate between the flowers of rape and wild turnip. Bees are known to often be loyal to one plant type on each foraging trip or over days (Proctor et al., 1996), due to the difficulty of interspecific pollen packing in the corbiculae (Zahavi et al., 1983). Honeybees are unable to distinguish between the two species on the basis of odor (Wright et al., 2002). Although flowers can appear very similar to the human eye, the UV reflectance patterns visible to insects may be quite different (Horovitz and Cohen, 1972, Proctor et al., 1996). This was established for rape and the wild turnip populations used in this study, which markedly differ in UV reflectance (Figure 16). To bees the flowers of these two species will appear completely different, and both species are very unlikely to be visited during a single foraging trip. The low frequency of interspecific hybrids that were obtained during bee pollination are likely to result from a few bees exhibiting “minoring” behaviour and switching species between foraging trips, or cross-contamination of pollen within the hive between bees working the different species (Ramsay et al., 1999).

In conclusion, both wind and bees can effect pollen-mediated gene flow from rape to wild turnip. The substantially lower frequency of hybrids from bee pollination relative to the more random wind pollination suggests that bees may be exhibiting some floral-constancy when foraging for pollen or nectar on
flowers of rape and wild turnip. The differing UV reflectance patterns from flowers of these two species could be the basis for this discrimination.
Chapter Five: Survival, establishment and fertility of interspecific brassica hybrids in an agricultural environment

5.1 Introduction

The introduction of genetic engineering as a plant breeding tool and the subsequent commercialization of transgenic crops have resulted in a rapid increase in the study of gene flow between agricultural and ruderal species (Conner et al., 2003, Messeguer, 2003). Transgenic crops with tolerance to herbicides and enhanced ability to resist the depredations of pests and diseases have already been widely commercialized, with a new array of transgenic crops with improved quality traits and the ability to produce industrial and pharmaceutical products anticipated to follow (Nap et al., 2003). As well as potentially enhancing the productivity of agriculture and reducing the environmental costs of production, there is a possibility that the new transgenes in these genetically modified crops may become established in closely related weed species (Crawley et al., 1993). This is perceived as having negative impacts resulting in new difficulties in the management of weeds, or ecological disturbances to ruderal populations of plants and secondary impacts on biodiversity (Conner et al. 2003, Ellstrand, 2003).

The use of genetic markers, either transgenic or naturally occurring, provides a valuable tool to investigate gene introgression between species. These can involve both molecular markers such as simple sequence repeats (Wilkinson et al. 2000), or morphologically unique or physiologically distinguishable classical markers to identify hybrid progeny (Jenkins et al., 2003; Manasse, 1992). The latter allow cost effective, quick and easy monitoring of large populations and simplify the quantification of gene introgression (Messeguer, 2003).

The introgression of genes between species is a multi-step process involving factors such as the sympatry of wild relatives, mating systems, sexual compatibility, efficiency and effectiveness of seed dispersal, and fitness of hybrid progeny (Conner et al., 2003; Messeguer, 2003). Gene flow studies in
brassica have focused on the frequency of interspecific hybridization (e.g. Jørgensen and Andersen, 1994; Scheffler et al., 1993; Bing et al., 1996; Chapter 3) or the ability of hybrids to form F2 or backcross generations (e.g. Bing et al., 1995; Chèvre et al., 1996; Hauser et al., 1998b; Halfhill et al., 2001; Chapter 6), while relatively few studies have examined the survival and fitness of hybrid plants in the field (Hauser et al., 1998a, Wilkinson et al., 2000).

This study investigated the fitness and survival of interspecific hybrids formed between five populations of wild turnip (Brassica rapa var. oleifera DC) and a herbicide-resistant rape (Brassica napus). The sites of three previous field experiments examining the frequency of interspecific hybridization between these species were monitored for interspecific hybrid seedlings. The fecundity of the interspecific hybrid seedlings was recorded. As well, a field site was sown with known mixtures of wild turnip populations and their interspecific hybrids with herbicide-resistant rape. The relative survival and establishment of the seedlings was monitored over eight weeks and the reproductive fitness of the surviving interspecific hybrids was determined following flowering and seed set.

5.2 Materials and methods

5.2.1 Interspecific hybrid establishment following open field pollination

Three trial sites from earlier field hybridization experiments (Chapter 3) were monitored for the establishment of interspecific hybrids between wild turnip and rape. Trial sites were randomized blocks of wild turnip populations, into which herbicide-resistant rape plants were introduced at flowering. The rape plants used were homozygous at a single locus for a dominant mutation conferring resistance to the herbicide chlorsulfuron. The rape plants were removed at the cessation of flowering, and therefore did not contribute to the seed bank.

The three trial sites were maintained under different management regimes. The first trial site remained fallow under grass for four years, and was subsequently cultivated in the spring. The second trial site was over-wintered and then cultivated in the spring. The third trial site was also over-wintered, then cleared.
of wild turnip plant debris and sprayed with glufosinate to remove all other plant species before the spring germination.

The density of wild turnip seedlings was estimated after the spring flush of seed germination. A quadrat $0.1\, \text{m}^2$ was used to estimate seedling numbers per $\text{m}^2$, the measurement repeated and the mean used. When the wild turnip seedlings had established to approximately the six true leaf stage, they were sprayed with Glean (a commercial preparation of chlorsulfuron) equivalent to $20\, \text{g.ha}^{-1}$ of chlorsulfuron. After three weeks putative interspecific hybrid plants were clearly discernable, as they were the only survivors.

All putative hybrids were subjected to flow cytometry to confirm their hybrid status by determining their ploidy level, using the methods described in Chapter 2. All interspecific hybrid plants were repeatedly examined over the remainder of the growing season in the field for the formation of open pollinated seed.

5.2.2 Interspecific hybrid establishment following sowing of seed mixtures

The seed used in this study originated from five populations of wild turnip (Massey, Riwaka, Marshland, Greymouth, and Makarewa; Table 2) following open pollination involving equal numbers of wild turnip and rape plants in a glasshouse by entomophily (Chapter 4). The rape plants were homozygous at a single locus for a dominant mutation conferring resistance to the herbicide chlorsulfuron. Seed from several wild turnip plants within each population were pooled to produce the required number of seeds. A one thousand seed aliquot (five samples of 200 seeds) was screened in vitro for chlorsulfuron resistance to determine the proportion of interspecific hybrids using the method described in Chapter 2.

Seed was broadcast sown in the field in spring time (November, 2002) at approximately 220 seeds per $\text{m}^2$ and lightly buried to a depth of approximately 5-10 mm by raking. There were five replicated plots, each $5\, \text{m} \times 6\, \text{m}$, with five populations in each plot. Each population was sown in a $1\, \text{x} \, 6\, \text{m}$ plot, with the arrangement of populations randomized among replicates. The population density of germinated seedlings was estimated 4 weeks after sowing, using the same quadrat method outlined above. The entire experimental area was
divided, perpendicular to the population plots, into three strips which were sprayed at two weekly intervals with Glean equivalent to 20 g.ha\(^{-1}\). Seedlings were sprayed at four weeks (3-4 true leaves), six weeks (5-6 true leaves) and eight weeks (initiation of bolting and flower buds). The number of seedlings surviving the herbicide treatment was recorded. The effects of source population and replication were examined using ANOVA. Hybrid survival at the different times was compared using t-tests. All surviving putative hybrids were repeatedly examined over the remainder of the growing season in the field for the formation of open pollinated seed.

5.3 Results

5.3.1 Interspecific hybrid establishment following open field pollination

The three trial sites were left for differing periods of time under different management regimes following the completion of earlier field experiments. A large number of seedlings germinated at all sites (Table 5), most of which were brassica seedlings. Some fathen (*Chenopodium album*) and white clover (*Trifolium repens*) seedlings were also observed, but these were at combined densities of approximately 2 seedlings per square metre and therefore did not impose competition on the brassica seedlings. They were susceptible to the chlorsulfuron spray. Seedling density varied from 272 to 673 seedlings per m\(^2\). The highest density of seedlings occurred at the site where the land had been fallow for 4.5 years, while the two other sites had been fallow for only 6 or 7 months. Only 1-3 herbicide-resistant seedlings were observed at each trial site. All of these herbicide-resistant seedlings were found to be triploid (Table 6), thereby confirming their interspecific hybrid status between wild turnip and rape. None of these interspecific hybrids set seed in the field (Table 6). The overall frequency of these interspecific hybrid plants ranged from 4.5 to 15.6 per 100,000 brassica seedlings, which is substantially lower than the frequency of 0.1 to 1.1 % interspecific hybrid seedlings originally harvested at each site (Table 5).
Table 5. Frequency of interspecific hybrids observed to survive in the field following open field pollination of wild turnip populations with a chlorsulfuron-resistant rape.

<table>
<thead>
<tr>
<th>Site</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date seed sown</td>
<td>Nov 1997</td>
<td>Nov 1998</td>
<td>Nov 2001</td>
</tr>
<tr>
<td>Date seed harvested</td>
<td>Mar 1998</td>
<td>Mar 1999</td>
<td>Mar 2002</td>
</tr>
<tr>
<td>Frequency of chlorsulfuron-resistant seedlings in harvested seed (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Date site sprayed</td>
<td>Sep 2002</td>
<td>Oct 1999</td>
<td>Sep 2002</td>
</tr>
<tr>
<td>Fallow time (months)</td>
<td>52</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Area sprayed (m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>36</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>Seedling density (number/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>623</td>
<td>272</td>
<td>535</td>
</tr>
<tr>
<td>Estimated number of seedlings sprayed</td>
<td>22428</td>
<td>10880</td>
<td>19260</td>
</tr>
<tr>
<td>Number of chlorsulfuron-resistant seedlings recovered</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Frequency of chlorsulfuron-resistant seedlings (%)</td>
<td>4.5 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>9.2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>15.6 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>These are from Chapter 3, and represent the seed deposited in the seed bank by the previous experiments.
Table 6. Flow cytometry analysis and fertility of putative interspecific hybrids established following open field pollination of wild turnip with pollen from herbicide-resistant rape

<table>
<thead>
<tr>
<th>Trial sites/samples</th>
<th>Genome size (pg DNA/2C nucleus*)</th>
<th>Fertility (seeds/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.86</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.86</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>1.87</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>1.90</td>
<td>0</td>
</tr>
<tr>
<td>3c</td>
<td>1.84</td>
<td>0</td>
</tr>
</tbody>
</table>

*Simultaneous measurements of the genome size of the two parent species produced values of 1.16-1.18 pg DNA/2C nucleus for wild turnip (CC; 2n = 2x = 18) and 2.55-2.57 pg DNA/2C nucleus for rape (AACC; 2n = 4x = 38).

5.3.2 Interspecific hybrid establishment following sowing of seed mixtures

The density of germinated seedlings ranged from 30-135 per m², which is low given the sowing density of approximately 220 seeds per m². ANOVA tests showed no differences in the frequency of hybrid seedlings between populations or replications (Table 7). Data were therefore pooled to increase sensitivity of statistical analyses, and the frequency of hybrids at the different times of herbicide treatment was compared using t-tests. The frequency of interspecific hybrids among the sown seed was determined by in vitro screening of seeds in the laboratory and ranged from 1.3-5.3% for the various populations. The herbicide treatment at the 4-6 true leaf stage in the field identified 1.5-4.0% of hybrids across the populations, which was similar to the range of 1.5-2.8% of hybrids identified following herbicide treatment at the 6-8 true leaf stage (Table 7). When the herbicide treatment was applied at the bolting stage or when flower buds were beginning to emerge, the frequency of hybrids had declined to between 0.9-1.2% for the various populations. A t-test determined no significant differences between the frequency of interspecific hybrid seedlings present in the brassica populations at the time of the first and second herbicide
treatments. However, the frequency of interspecific hybrids had significantly declined between the times of the second and third, and the first and third herbicide treatments. A correlation analysis was performed on the data collected from 4 to 8 weeks after sowing and showed that both plants counts and frequency of interspecific hybrids decreased significantly over time (p<0.001 and p=0.003 respectively). This decline was not affected by population. All the surviving interspecific hybrids failed to set seed in the field, despite the presence of numerous wild turnips plants flowering at the same time in the neighbouring environment.
Table 7. Original wild turnip x herbicide-resistant rape interspecific hybrid frequency and survival of interspecific hybrids derived from five wild turnip populations sown in mixtures in an agricultural environment

<table>
<thead>
<tr>
<th>Populations of wild turnip</th>
<th>Original frequency in seed sown</th>
<th>Four weeks after sowing (4-6 true leaves)</th>
<th>Six weeks after sowing (6-8 true leaves)</th>
<th>Eight weeks after sowing (initiation of bolting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Massey</td>
<td>3.19</td>
<td>2.01</td>
<td>1.49</td>
<td>0.92</td>
</tr>
<tr>
<td>Riwaka</td>
<td>5.29</td>
<td>4.03</td>
<td>2.34</td>
<td>0.95</td>
</tr>
<tr>
<td>Marshland</td>
<td>3.96</td>
<td>1.84</td>
<td>2.78</td>
<td>1.03</td>
</tr>
<tr>
<td>Greymouth</td>
<td>1.29</td>
<td>2.87</td>
<td>2.08</td>
<td>1.23</td>
</tr>
<tr>
<td>Makarewa</td>
<td>5.26</td>
<td>1.50</td>
<td>2.72</td>
<td>0.89</td>
</tr>
<tr>
<td>Pooled data</td>
<td>3.80</td>
<td>2.45</td>
<td>2.28</td>
<td>1.00</td>
</tr>
</tbody>
</table>

There were no differences between replication ($F=0.235-1.911$, $df=4,16$, $P=0.547-0.914$) or populations ($F=0.096-0.724$, $df=4,16$, $P=0.588-0.982$) within each time period. T-tests on pooled data showed that the frequency of interspecific hybrids after four weeks was not significantly different from that observed after six weeks ($t=0.332$, $df=24$, $P=0.742$). There were significant differences following the herbicide treatments at four and eight weeks ($t=3.338$, $df=24$, $P=0.003$), and at six and eight weeks ($t=3.083$, $df=24$, $P=0.005$).
5.4 Discussion

It is apparent from previous studies, both in this thesis (Chapters 2, 3 and 4) and from other publications (e.g. Palmer, 1962; Stace, 1991; Jørgensen and Andersen, 1994; Bing et al. 1996) that rape and wild turnip have the ability to produce interspecific hybrids. The next step in establishing the likelihood of introgression of genes from rape to wild turnip populations is to determine the ability of interspecific hybrids to survive in the field environment. This study investigated the establishment of interspecific hybrid seedlings in competition with other brassica seedlings in an agricultural environment.

The first component of this study consisted of observations of the brassica weed seed bank established following previous field experiments investigating the incidence of hybridisation between rape and wild turnip (Chapter 3). Three field sites were subjected to two management regimes. One site was left for three years under grass and then cultivated. This site had the greatest density of brassica seedlings and was therefore likely to have contributed the greatest number of interspecific hybrid seeds to the soil seed bank. The higher seedling density is unlikely to be related to the amount of time that the land was left fallow, but rather to the higher seed number produced per plant in the original experiment (Figure 10). Germinated seedlings were predominantly wild turnip, with two other weed types occurring at low densities. The two other field sites were left fallow for only one season, before seed germination the following spring. All field sites had rare interspecific hybrid seedlings occurring among a very large population of wild turnip (Table 5). At all three sites the frequency of interspecific hybrid seedlings was several orders of magnitude less than expected given the frequency of interspecific hybrid seedlings found at the time of seed harvest from each field site (Table 5).

These rare interspecific hybrids between rape and wild turnip were identified by their resistance to a field application of a herbicide (chlorsulfuron) after germination of seeds from the seed bank resulting from the field pollination of wild turnip and a herbicide-resistant rape. The rape plants did not contribute to the seed bank since
they were introduced to the field sites as potted plants just prior to flowering and were removed from the field after flowering ceased (Chapter 3). Flow cytometry was used to confirm that the chlorsulfuron-resistant brassica plants recovered from the field sites had the triploid status expected for interspecific hybrids between wild turnip and rape (Table 6). This triploid status was further verified by the infertility of these plants (Table 6).

The second component of this study involved a replicated field experiment designed to further investigate the above field observation and verify the survival and fitness of interspecific hybrids between wild turnip and rape in an agricultural environment. Wild turnip populations used in this study have shown differences in seed production, and differences between populations in the frequency of interspecific hybrid progeny produced with rape (Chapter 2), but this effect has not been apparent in the field. This study showed there were no differences between wild turnip populations in the ability of their interspecific hybrids with rape to germinate and survive under field conditions (Table 7). The brassica populations emanating from the seed bank established after the earlier experiments were between 272 and 623 seedlings per square metre (Table 5), while the deliberately sown trial resulted in between 30 and 135 seedlings per square metre. The relatively low emergence rate in the field trial resulted in little competition between the hybrids and the wild turnip seedlings. The gradual decrease in the frequency of interspecific hybrid seedlings over eight weeks from sowing (Table 7) is unlikely to have resulted from direct competition with the wild turnip seedlings, but rather from an overall reduced plant vigour.

The number of interspecific hybrid seedlings that survived in the field environment is substantially lower than the number of interspecific hybrid seedlings initially present in the natural seed bank (Table 5) or deliberately sown in the field (Table 7). Based on the estimated number of individual interspecific hybrids there was a loss of 98% during establishment from the natural seed bank and a loss of 74% over eight weeks from intentional sowing of a mixed population of wild turnips and wild turnip x rape interspecific hybrids. The likelihood of gene introgression from
rape to wild turnip is further reduced by the lower levels of fitness in these hybrids. The failure of these hybrid plants to set seed in the field was not limited by pollen availability since there were a large numbers of wild turnip pollen donors present at the edge of all herbicide-treated areas. This reduction in fertility may have arisen from the herbicide treatment of the interspecific hybrids in the field. However, the data presented in Appendix 1 demonstrate that interspecific hybrids grown in the field without herbicide applications had similar low fecundity to those treated with the herbicide in this study. Further investigations reporting the low fertility of the interspecific hybrids under glasshouse conditions are presented in Chapter 6.

Conspecific hybridisation of inbred lines often results in a high degree of heterosis with consequent gains in plant vigour and yield, especially in cross pollinating species (Allard, 1960). Such heterosis is also well known following hybridisation between some individuals within and between natural populations (Darwin, 1876, Grant, 1981). Interspecific hybrids vary in fitness, from those that have poor survival and reproduction, to those that may equal or surpass their parent species in plant vigour, establishment and fitness (Allard, 1960; Raven 1976; Grant, 1981). Progeny from some interspecific crosses between cultivated forms of *B. napus* and *B. rapa* have been previously recorded to exhibit heterosis for biomass yield, although there are strong genotypic effects with some combinations resulting in reduced biomass (Lu *et al.*, 2003; Qian *et al.* 2003). F₁ hybrids between *B. napus* and weedy Danish populations of *B. rapa* have been reported to be highly fit (Hauser *et al.*, 1998a). Rape seed has low soil persistence (Lutman, 1993), and has no dormancy when initially ripened and deposited in the soil, but can develop secondary dormancy (Pekrun *et al.*, 1998). Linder (1998) reported poor performance of canola x wild turnip hybrid seed, which lacked any dormancy. The data from this study suggest hybrids between New Zealand wild turnip populations and *B. napus* do not exhibit hybrid vigour, and in fact have a highly reduced fitness and fertility. The marked contrast between this study and that of Hauser *et al.* (1998a) may be a consequence of the different *B. rapa* subspecies used. Although the subspecific status of *B. rapa* is not given by Hauser *et al.* (1998a and b), other studies from the same research group state *B. rapa* ssp. *campestris* (Jørgensen
and Andersen, 1994). Alternatively it may represent a differentiation of the plant populations between Europe and New Zealand.

The apparent lower survival, vigour and fitness of interspecific hybrids between wild turnip and rape relative to the parent species are important factors that should be taken into account when evaluating the gene flow risks associated with the release of genetically modified plants.
Chapter 6: Fertility and inheritance of herbicide resistance in interspecific hybrids between wild turnip and rape.

6.1 Introduction

The successful escape of transgenes from crops into wild populations of related species requires that the interspecific hybrids are fertile. For introgression of genetic material, backcrossing to a wild plant population is especially important. This is most likely to occur with the interspecific hybrid as the maternal parent since potential chromosome imbalance is likely to result in severe pollen competition (Khush 1973). Stable introgression could result in genetically enhanced weeds (Hansen et al., 2001), which may alter the ecology and management of the ruderal and cultivated environment. Wild turnip is a highly successful weed (Holm et al., 1997), while rape struggles to survive outside cultivation (Crawley et al., 2001; Heenan et al., 2004). The potential of transgene introgression from cultivated rape to wild turnip may further enhance weedy attributes. Wild turnip has primary dormancy, while interspecific hybrids with rape inherit the secondary dormancy characteristics of the rape parent (Landbo & Jørgensen, 1997). Introgression of transgenes from rape into the wild turnip genome will potentially allow these genes to reappear in the genetic landscape for years via the seed bank (Linder & Schmitt, 1995, Landbo & Jørgensen, 1997).

Rape is highly self-fertile (Free & Nuttall, 1968), whereas wild turnip is self-incompatible (Williams, 1978; Holm et al., 1997). If interspecific hybrids inherit the self-incompatibility of the wild turnip parent, they will be obligate outcrossers. Therefore, they will need to grow in the presence of other compatible plants in order to successfully complete sexual reproduction and for gene introgression to occur. Hybrid seedlings frequently have low field survival (Stebbins, 1958; Chapter 5), which reduces the likelihood of introgression of genes, but high hybrid fitness of mature plants may increase gene survival.
The inheritance pattern of genes following interspecific hybridisation plays an important role in the success of gene introgression between species. Regular Mendelian inheritance patterns will allow rapid and easy introgression into weedy populations. Unbalanced chromosome transmission resulting in skewed segregation could either increase or decrease the frequency of gene transmission depending on selection for or against a specific trait or closely linked loci.

Previous work in this project has examined the potential for interspecific hybridisation to occur between wild turnip and rape using hand pollination (Chapter 2) and in the ‘real world’ of open field pollination (Chapter 3). Further experiments looked at the ability of the interspecific hybrid seeds to survive in the seedbank, and the ability of hybrid seedlings to establish and survive in the field (Chapter 5). This study investigates the fertility of the interspecific hybrid plants and their ability to sexually reproduce. For successful introgression of genes into a wild population, the ability to backcross to the wild turnip parent is important. If an interspecific hybrid is able to self pollinate either within an individual plant or with another hybrid plants, a gene is likely to have further opportunities over several seasons to successfully introgress into wild populations.

6.2 Materials and methods

6.2.1 Plant material

Seed used in this study was derived from the open pollination of wild turnip as the maternal parent with rape plants homozygous at a single locus for a dominant mutation conferring resistance to the herbicide chlorsulfuron. The wild turnip plants were from five New Zealand populations of a wide geographic range (Massey, Riwaka, Marshland, Greymouth, and Makarewa; Table 2). This seed was screened in vitro for chlorsulfuron resistance to identify interspecific hybrids using the method described in Chapter 2 (Section 2.2.3). Ten interspecific hybrids derived from each wild turnip population were subjected to flow cytometry (Section 2.2.4) to confirm their triploid status, then potted into individual 8 litre pots of standard potting mix (as described in Section 4.2). Eight rape seedlings, isogenic
to the original rape parent but without chlorsulfuron resistance and ten seedlings from each of the five wild turnip populations were grown in the same conditions. At flowering six plants were selected from each population to be used as maternal plants, and as pollen donors. All plants used in the experiment were of similar size and maturity.

6.2.2 Pollen viability

Pollen from 3 flowers from each plant used as a pollen donor were collected and the fertility of the pollen tested using Alexander’s stain (Alexander, 1969). Pollen from each flower was smeared on a microscope slide, and a drop of stain added. Staining was observable within a minute, with viable pollen staining red and inviable pollen staining pale green/blue. Pollen was counted until at least 100 stained grains had been counted for each flower.

6.2.3 Pollen germination

Using 24-well plates, pollen from one flower was placed in a single well. Approximately 250-300 µl of pollen germination medium (Shivanna and Sawhney, 1993) was dispensed into each well. After 2 hours, each well was examined under an inverted microscope, and germinated pollen grains recorded. If pollen tube length was half the diameter of the pollen grain or longer it was counted as germinated. For each flower 100-150 pollen grains were scored, and three flowers from each plant tested.

6.2.4 Hand pollinations

Individual flowers from each herbicide-resistant interspecific triploid hybrid plant (AAC) were opened while at the yellow bud stage, emasculated and pollinated with mature pollen. Pollen sources involved self pollinations using pollen from another flower of the same plant (AAC) and pollen from another hybrid plant derived from the same wild turnip population (AAC), and backcrosses using pollen from the appropriate original wild turnip population (AA) and the isogenic herbicide-sensitive rape plants (AACC). Immediately following pollination the flowers were capped
with aluminium foil and left for one week, after which the cap was removed and the silique left to develop. Four replicate pollinations were performed for each cross on each plant.

6.2.5 Seed production

A successful pollination was recorded when a silique containing seeds was formed. When the siliques were mature, they were harvested and the number of seeds recorded within each silique.

6.2.6 Inheritance of herbicide resistance

Seed was germinated on the same medium previously described for screening hybrid seedlings (Section 2.2.3). The number of seedlings with chlorsulfuron resistance and sensitivity were scored based on root length.

6.2.7 Analysis performed

The effects of maternal wild turnip population on percentage of pollen viability and in vitro pollen germination were analysed using ANOVA with individual plants treated as replicates. The cumulative totals of pollination events resulting in siliques with and without seed for the various sources of pollen were compared using Chi-square tests of independence. The segregation of herbicide resistance in the selfed and backcrossed progeny were tested against the expected Mendelian segregation using goodness-of-fit Chi-square tests.

6.3 Results

6.3.1 Pollen fertility of hybrids

The use of Alexander’s stain for assessing pollen viability clearly showed a substantial reduction in pollen fertility of the triploid interspecific hybrids relative to the parental species (Figure 17). In contrast to the 100% pollen viability of both parent species, the mean pollen viability from interspecific hybrid plants derived from all wild turnip populations ranged from only 35-41%.
The results from *in vitro* pollen germination were even more dramatic. Although pollen germination of both parents was virtually 100%, the mean pollen germination from hybrid plants derived from all populations of wild turnip was only 13-17% (Figure 18). There were no significant differences between wild turnip populations the interspecific hybrids were derived from for either pollen viability assessed by Alexander's stain (Figure 17) or for *in vitro* pollen germination (Figure 18).

![Figure 17. The fertility of wild turnip x rape hybrids, and rape and wild turnip species as shown by Alexander's Stain. There were no significant differences between the hybrids derived from different maternal wild turnip populations (F=0.59, df1=4, 24, P=0.67). Error bars represent standard errors.](image)
Figure 18. The in vitro pollen germination of wild turnip x rape hybrids, and rape and wild turnip plants. There were no significant differences between the maternal wild turnip populations ($F = 0.16$, $df_1 = 4, 24, P = 0.96$). Error bars represent standard errors.

6.3.2 Hybrid fertility as measured by seed production

Pollination success as measured by the production of siliques with seed is presented in Figure 19. Overall, four hundred and eighty pollinations were performed, and of these 424 (88%) failed to set any siliques or seed. There were no differences in the frequencies of silique development with seeds on the basis of the wild turnip population used as the maternal parent for the interspecific hybrid ($\chi^2 = 1.911$, $df = 4, P = 0.752$). Self pollination of the triploid interspecific hybrid plants resulted in the similar lack of success when crossing within an individual plants or
between hybrid plants derived from the same maternal wild turnip population ($\chi^2=1.670, df = 1, P= 0.196$). There were no significant differences between the backcrosses to rape and wild turnip ($\chi^2= 1.296, df=1, P=0.255$). Backcrossing using pollen from rape and wild turnip plants was significantly more successful than self-pollination for producing siliques with seed on the triploid interspecific hybrids (Figure 19). For successful production of siliques with seed there were significant differences between self pollination within plants and backcrosses with rape or wild turnip pollen ($\chi^2= 13.706, df=1, P<0.001$ and $\chi^2=7.053, df=1, P=0.008$ respectively). There were also significant differences between self pollination within populations and backcrosses to rape or wild turnip ($\chi^2= 21.943, df=1, P<0.001$ and $\chi^2=13.897, df=1, P<0.001$ respectively).

Most siliques contained one seed. Of the successful pollinations, 44 produced one seed (79%), nine produced two seeds (16%), two produced three seeds (5%) and one fecund pollination resulted in 13 seeds (2%). The mean number of seeds per successful pollination were not formally compared between pollen types due to the paucity of successful pollinations. The single highly successful pollination event meant that within plant self-pollinations were more successful than within hybrid population self-pollinations (Figure 20). Backcrossing to both parents produced similar amounts of seed to within hybrid population. Source of pollen effected the production of seed, but this result is possibly biased by stochastic events arising from the one self-pollinated event that produced 13 seeds.
Figure 19. Pollination success as measured by the presence of siliques.
6.3.3 Segregation of herbicide resistance in progeny of interspecific hybrids

Since the number of seeds produced was very low, the data from all plants in all populations was pooled to assess inheritance of herbicide resistance in the progeny (Table 8). It was expected that self-pollinations would produce progeny in a 3:1 ratio of susceptible to resistant to herbicide, if the herbicide resistance gene was inherited in a standard Mendelian manner. Backcrossing was expected to produce a ratio of 1:1 susceptible to resistant progeny. The inheritance of herbicide resistance was regular in backcrosses, but highly skewed in self pollinations with a excess of herbicide-sensitive progeny (Table 8).
Table 8. Segregation of herbicide resistance in progeny populations from triploid interspecific hybrids of rape and wild turnip. Data is pooled from all plants derived from each of the wild turnip populations

<table>
<thead>
<tr>
<th>Cross (♀x ♂)</th>
<th>Observed data</th>
<th>Expected ratio</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>HS</td>
<td></td>
</tr>
<tr>
<td>Hybrids x hybrids</td>
<td>10</td>
<td>14</td>
<td>3:1</td>
</tr>
<tr>
<td>Hybrids x rape</td>
<td>14</td>
<td>13</td>
<td>1:1</td>
</tr>
<tr>
<td>Hybrids x wild turnip</td>
<td>17</td>
<td>8</td>
<td>1:1</td>
</tr>
</tbody>
</table>

6.4 Discussion

A high male fertility in brassica plants, as with any other plant, greatly enhances the likelihood of genetic material being passed to subsequent generations. Interspecific hybrids plants between rape and wild turnip are triploids with a genome construction of AAC. In such hybrid plants the single locus with herbicide resistance is carried in a heterozygous state on either the C genome or one of the A genomes. If pollen fertility is low, and Mendelian segregation is irregular then the chances of successful introgression are significantly reduced. The pollen of the rape and wild turnip parents was highly fertile, with Alexander’s stain showing 100% pollen viability (Figure 17), and virtually 100% in vitro pollen germination (Figure 18). In contrast, the pollen from the interspecific hybrids contained many non viable pollen (Figure 17) and even less pollen capable of germination (Figure 18). Although it is not impossible for this pollen to produce progeny with the herbicide resistance gene, its low fertility and poor germination makes this highly unlikely.

The pollen produced by interspecific hybrids has been shown to be less fertile than the pollen from the parent species in many cases (Stace, 1975). Hauser et al.
(1998) reported that F₁ hybrid pollen of *B. rapa* and *B. napus* is approximately 46% fertile, while *B. rapa* was 91% fertile and *B. napus* was 98% fertile. Jørgensen et al. (1996) reported hybrid pollen fertility to be 35% on average. The results presented here show interspecific hybrids between rape and wild turnip populations from New Zealand to have markedly reduced male fertility (Figure 17 and Figure 18), and considerable higher infertility than the hybrids tested by Hauser et al. (1998) or Jørgensen et al. (1996).

Seed production was very low, with most pollination events resulting in neither silique development nor seed formation (Figure 19 and Figure 20). Total seed produced was similar for the self-pollinations and the backcrosses to the two parental species (Figure 20), and was overall extremely low reflecting the low female fertility of the interspecific hybrids. Most pollinations (88%) did not result in a silique, further reflecting the low female fertility of the hybrids. F₁ plants from interspecific crosses often have low female fertility (Grant, 1981, Stace, 1975), but this can vary depending on environmental factors and different genes (Ellstrand, 2003). Although highly fit F₁ hybrids between *B. napus* and *B. rapa* have been reported (Hauser et al., 1998, Jørgensen et al., 1996), the hybrids of all populations of rape and wild turnip will not necessarily behave in the same manner. This has been well illustrated by interspecific hybridization between 20 populations of *Salvia apiana* and *S. mellifera* (Meyn & Emboden, 1987).

Of the 424 individual pollination events attempted, 88% failed to set seed. For the few successful pollination events, all but one produced only three or less seeds. A single self-pollination event produced thirteen seeds, all of which proved to be susceptible to herbicide. Isolated pollination events that result in large numbers of seed may be important in the introgression of genes, but in this case the single event did not progress introgression of the herbicide resistance.

Normal chromosomes segregation appears to occur in maternal gametes of the interspecific hybrids. This is indicated by the backcrosses progeny arising from pollen of both parent species segregating in the expected 1:1 ratio (Table 8). In contrast, self-pollinations give an excess of herbicide-sensitive progeny suggesting
that transmission of herbicide-resistance is poor in pollen (Table 8). This is not unusual since pollen with unbalanced chromosome numbers is well known to perform poorly (Khush, 1973). This is supported by the poor pollen viability and in vitro pollen germination (Figure 17 and Figure 18). Aneuploid pollen from triploid plants is also expected to be out-competed by any rare pollen that have by chance reverted back to haploid status based only on the A genome through chance segregation of chromosomes. The observed numbers of herbicide-resistant progeny found in self-pollinations (Table 8) fits a 1:1 ratio ($\chi^2=0.66$, df=1, $P>0.05$). This suggests that there is no transmission of herbicide resistance through the pollen, which might be expected if the herbicide resistance locus was on the C genome. However, this can not be confirmed without reciprocal backcrosses being screened, which is difficult given the poor pollen fertility.

These results from New Zealand populations are in marked contrast to data from Danish populations. Interspecific hybrids between *B. napus* and *B. rapa* are reported as being more fit than the parental species, as the individual siliques contain fewer seeds, but the plant 'compensates' by producing a greater number of siliques overall (Hauser *et al.*, 1998b). Unexpectedly, the progeny from these F$_2$ interspecific hybrids showed lower fitness than the F$_1$ hybrids (Hauser *et al.*, 1998a). This illustrates the importance of making risk management decisions based on in-depth studies rather than single season one generation experiments.
Chapter 7: Application of @risk to model the likelihood of introgression of transgenes from GM rape to wild turnip and rape populations.

7.1 Introduction

Predictive mathematical modeling allows researchers to use existing ecological data to estimate the likelihood of possible future scenarios (Hails et al., 2002). A concern with the introduction of genetically modified plants involves gene flow to related species and the potential increase in their weediness (Bullock, 1999). The impacts of introducing genetically modified plants will be better assessed by using scientifically robust approaches to analyse the frequencies of genes introgression and enhanced fitness to the resulting plants. One approach to estimating the extent of this increase is to perform multiple experiments over a range of habitats (e.g. Parker and Kareiva, 1996, Snow and Palma, 1997). Such exhaustive studies were conducted by Crawley et al. (2001) in four specific crop examples.

Weediness, as measured by the increase in a weed population over time has been shown to be habitat specific (Bullock, 1999). Consequently, any data is highly context dependent and few generic conclusions can be drawn. Undertaking large scale gene flow assessments in a wide range of habitats would be prohibitively expensive and difficult to justify. Alternative approaches including a modeling component are required (Kareiva, 1990). Sensitivity analysis of parameters contributing to gene flow in particular situations can be used to develop management techniques targeting the most vulnerable stage of gene introgression and thereby minimizing gene flow (Kareiva et al., 1996). The situations modeled can be specific to a particular crop and weed pair or a specific environmental niche (Gillman and Hails, 1997).

Models predicting gene flow have been previously developed for various reasons. The model GENESYS was developed to rank cropping systems by their risk of gene dispersal, the model having been specifically developed for winter oilseed rape.
interbreeding with rape volunteers (Meynard et al., 1999; Colbach et al., 2000ab). This model incorporated a large range of factors, including spatial dimensions, and was tested and applied by Colbach et al. (2001b). It focused on the likelihood of intraspecific gene flow between agricultural and wild rape plants, and specifically examined the effects of mowing roadside verges and set-aside areas, and the influence of field shape. They reported that mowing is important to reduce transgene dispersal in the ruderal environment, and that roadside and set-aside management has an important effect on transgene establishment in the ruderal and agricultural environments (Colbach et al., 2001a and b).

A stochastic model attempts to reflect the inherent variation of biological systems (Gilman and Hails, 1997). By using real data to define the limits and forms of stochasticity, a realistic picture which still incorporates random variation can be created. Models can be used to refine and direct further field work by revealing the most sensitive demographic transition (Bullock, 1999). Different scenarios can be modeled, such as varying the initial population size and using the model to examine the effects of the change on gene flow in subsequent seasons (Gilman and Hails, 1997). Particular aspects of gene flow can be modeled, such as pollinator effects on gene flow (Cresswell, 1994, 2003; Cresswell et al., 2002), or the effects of transgenic plants on other organisms (Watkinson et al., 2000).

A stochastic model of gene establishment in two fish species was developed by Davis et al. (1999) which clearly demonstrated the usefulness of predictive modeling to regulators and scientific research funding bodies. The rate of introgression of a gene was estimated for carp, which is a long-lived species and compared to the introgression rate of mosquito fish, which rarely survives the winter. Both species are significant pest fish in Australia. The rate of gene introgression for carp was significantly slower, and the model provided useful information to pest fish managers considering funding for transgenic control schemes (Davis et al., 1999).

A matrix model demonstrating the use of elasticity analysis was developed by Bullock (1999). The model examined the demographic parameters that would
change weediness. Projection matrices for a crop and three weeds were developed. Early use of models in long term scientific research on effective control of populations could be used to ascertain the factors most influential on model outputs. The information could be used to focus efforts on the most vulnerable phase of gene introgression, and reduce the gene dispersal risk of novel crops (Bullock, 1999).

Stochastic predictive statistical models usually involve several independent algebraic equations each a combination of variables with independent estimated distribution functions (for example Squire et al., 1997, and Davies et al., 1999). These equations and distributions are usually then combined using Monte Carlo simulations. Several computer programs have been used to run such simulations, including Turbo PASCAL (Cresswell et al., 1995), SPSS and STAGECOACH used by Bullock (1999). This project uses @risk (Palisade Corporation, 2002), an Excel spreadsheet add-on. @risk has been developed primarily for use as a tool for predicting financial scenarios using stochastic statistical models, but also has considerable potential in engineering and science (Palisade Corporation, 2002). No previous examples utilizing @risk for modeling biological situations could be traced; therefore, this thesis may be the first example of its use for ecological applications.

Previous work in this thesis and by others (eg Jørgensen and Andersen, 1994; Bing et al., 1996; Palmer; 1962; Wilkinson et al., 2003) has shown that there is potential for genes to transfer from rape to wild turnip. Chapter Two demonstrates that while there are interspecific barriers to introgression of genes from rape to New Zealand wild turnip populations, hybridization can readily occur. Chapter Three examines the frequency of interspecific hybrid production in the natural environment over several years. Chapter Four shows the relative importance of entomophily and anemophily to pollen movement between the species. In Chapter Five, the ability of interspecific hybrid seeds to survive winter burial, to successfully germinate, and for the resulting seedlings to survive in competition with wild turnips over the establishment phase is quantified. Chapter Six studies the male fertility
and the ability of the hybrids to set seed in backcrosses and produce F₂ progeny. Collectively, these previous chapters investigate the importance of key parameters associated with gene introgression from rape to wild turnip. This chapter seeks to accumulate most of this data, as well as data from other sources into a mathematical model to quantify and predict the rate of introgression of transgenes from rape to wild turnip, and identify the most sensitive steps along the introgression pathway. Control of gene flow is an important aspect of the management of transgenic crops in particular (Champolivier et al., 1999).

These data are used to generate input distributions for predictive statistical models. These are combined and simulations run using @risk to identify opportunities for more effective management of transgene introgression from *B. napus* to *B. rapa*. A key aim of this chapter is to demonstrate the relative ease and enormous power of @risk in modeling scenarios of genetic population change.

![Figure 21. Large populations of wild turnip can occur on disturbed land](image-url)
The model gave three output values after each simulation. The outputs were the number of interspecific hybrid progeny backcrossed to wild turnip, backcrossed to rape and third generation interspecific hybrids plants that survived to flowering in the third generation.

Several assumptions were made in the model. It was assumed that

- There was complete synchrony of flowering between rape and wild turnip
- That seedling survival and seedling emergence is the same for third generation plants as it is for second generation plants
- That most pollen is deposited in the three metre radius around the paternal plant as described by Lavigne et al. (1998)
- That wild turnip populations are the same size each generation

7.2 Input variables for modeling gene introgression

When considering input variables to model gene introgression from rape to wild turnip, the life history parameters that need to be considered involve both parental species as well as that of the interspecific hybrids. As each season involves a different group of plants as the interspecific and backcross plants become established in the environment, the process is better described as a linear progression, rather than a life cycle (Figure 22). The abbreviated outline view does not show all inputs used in the model, or all the calculations performed.
Figure 22. An abbreviated outline view of model. ih is an abbreviation for interspecific hybrid.
7.2.1 Variables derived from plant measurements

Input variables used in the model are defined in Table 9. Most of these are experimentally derived, and are specific to populations from New Zealand. The range and distribution of likely possible values used in the model is presented in summary form.
<table>
<thead>
<tr>
<th>Life History Parameter</th>
<th>Input variable</th>
<th>Mean</th>
<th>Distribution type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild turnip population size (year 1)</td>
<td>w1n</td>
<td>10</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Seeds per silique</td>
<td>s</td>
<td>10</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Proportion of wild turnips pollinated by rape</td>
<td>rp:p</td>
<td>0.01</td>
<td>Normal, $\sigma$=0.01, truncated to 0.0</td>
</tr>
<tr>
<td>Proportion of seed that germinates</td>
<td>se</td>
<td>0.25</td>
<td>Uniform, set with range of 0.09 to 0.41</td>
</tr>
<tr>
<td>Proportion of seed that survives</td>
<td>ss</td>
<td>0.5</td>
<td>Uniform, set with range of 0.4 to 0.6</td>
</tr>
<tr>
<td>Number of flowers on wild turnip plants</td>
<td>wf</td>
<td>245</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Number of flowers on rape plants</td>
<td>rf</td>
<td>245</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Number of flowers on interspecific hybrid</td>
<td>if</td>
<td>125</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Wild turnip population size (year 2)</td>
<td>w2n</td>
<td>10</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Rape population size (year 2)</td>
<td>r2n</td>
<td>5</td>
<td>Normal, $\sigma$=1</td>
</tr>
<tr>
<td>Pollen grain production for each wild turnip flower</td>
<td>wfp:f</td>
<td>18,000</td>
<td>skewed/custom, min=2000, max=20000 Figure 23</td>
</tr>
<tr>
<td>Pollen grain production for each rape flower</td>
<td>rfp:f</td>
<td>36,000</td>
<td>skewed/custom, min=2000, max=38000 Figure 23</td>
</tr>
<tr>
<td>Pollen grain production for each interspecific hybrid flower</td>
<td>ifp:f</td>
<td>27,000</td>
<td>skewed/custom, min=2000, max=28000 Figure 23</td>
</tr>
<tr>
<td>Proportion of pollen produced by interspecific hybrids that is viable and fecund</td>
<td>ipg</td>
<td>0.2</td>
<td>Uniform, set with range of 0.15 to 0.25</td>
</tr>
<tr>
<td>Seed set in interspecific hybrid flower after pollination with wild turnip pollen</td>
<td>if2s(wp)</td>
<td>0.23</td>
<td>Discrete, Table 10</td>
</tr>
<tr>
<td>Seed set in interspecific hybrid flower after pollination with wild type rape pollen</td>
<td>if2s(rp)</td>
<td>0.28</td>
<td>Discrete, Table 10</td>
</tr>
<tr>
<td>Seed set in interspecific hybrid flower after pollination with interspecific hybrid pollen</td>
<td>if2s(ip)</td>
<td>0.01</td>
<td>Discrete, Table 10</td>
</tr>
</tbody>
</table>

The sources of the data used for means and defining input variability are stated in the accompanying text. The input variable abbreviations are used in the model. Means presented here are those used in the first scenario modeled. Changes used in subsequent models are delineated in the text.
Number of wild turnip plants present in recipient populations (\(w1n\) and \(w2n\))

Canterbury was surveyed in September and October in 2003 for the abundance of *Brassica* taxa (Heenan et al., 2004). Wild turnip most commonly occurred in populations smaller than 10 plants with 67% of the observed populations being less than 10 plants. The uncertainty is represented by a normal curve (\(\sigma=1\)) in the model. This number was also used for the second year/generation, with the uncertainty represented by the same curve. It was assumed that the wild turnip populations would be a consistent size each year.

Seed produced per silique (s)

Rape produces 1-30 seeds per silique in intraspecific crosses (Clarke, 1979). Wild turnip produces 0-16 seeds per silique in interspecific crosses with rape (Chapter 2), and 1-20 seeds per silique in intraspecific crosses (Korpela, 1988). For the purpose of this study, a mean of 10 seeds per silique was used, with a uniform distribution and a range of 0-20 to reflect the wild turnips exposure to intraspecific and interspecific pollen.

Frequency of wild turnip pollinations by rape (rp:p)

Data is available for the frequency of interspecific hybrid seed set from the field trials at ratios of 400:1 and 1:1 (Chapter 3), where the highest rate of hybridization generated in these trials was 2.1% and the lowest was approximately 0.1% (Figure 7). These numbers were used to generate a mode of 1%, and for the purposes of this study set with a normal distribution (\(\sigma=0.01\)). This was truncated to prevent negative values being used in the model (min=0).

Interspecific hybrid seedling establishment the following season (se)

Using the data of average seed production per plant from Chapter 3 (Figure 10) and knowing the approximate number of plants in each trial (estimated in the methods of Chapter 3), an estimate can be produced of the total seed deposit by the original experiment (minus that used for data analysis). The density of
seedlings germinating from these trials was calculated in Chapter 5, giving data describing the survival and germination of seed from the seed bank. The proportion of germination for each of the three trials was 0.09, 0.14, and 0.40. A range of 0.2 to 0.3 was used in the model, with a uniform distribution of risk.

*Interspecific hybrid seedling survival to flowering (ss)*

Half of the hybrid seedlings that established in the field, died before flowering (Chapter 5). An arbitrarily set uniform distribution was placed on this factor, with a range of 0.4 – 0.6.

*Flowers per plant (wf, rf, if)*

The mean number of flowers per plant is set at 245, $\sigma=1$, for wild turnip and rape. This number is taken from Lefol et al. (1996), who examined three varieties of oilseed rape and found a range of 231-260 flowers per rape plant (statistical analysis showed there were no significant differences between the varieties). Although the number of flowers for each of the two plant species is assumed to be the same, they are entered into the model separately, as otherwise they became excessively important in the calculation of the outcomes. Interspecific hybrids are not as floriferous, and the arbitrarily chosen number of 125 flowers with a normal distribution ($\sigma=1$) is used.

*Rape population size in second generation (r2n)*

In the second year it is assumed that rape is not planted in the same paddock, but that there are 5 rape volunteers close enough to the wild turnip and hybrid population to be able to contribute to pollination. This number is based on the observations of Heenan et al. (2004) on the size of ruderal rape populations in Canterbury, and is set with a normal probability function ($\sigma=1$).

*Pollen production (wpf:f, rfp:f and ifp:f)*

Hauser et al. (1997) reported wild turnip contained approximately half the number of pollen grains per flower compared to rape. Each rape flower (rfp:f) is assumed
to produce a mean of about 36,000 pollen grains, as described by Cresswell et al. (2001), therefore about 18,000 pollen grains per flower is used for wild turnip pollen production (wfp:f) in this model was 18,000. Although most flowers would produce these numbers of pollen grains, in periods of extreme weather pollen production may be substantially less due to stress conditions. To reflect this, a custom distribution was created which shows the most likely pollen production to have means of 18,000 or 36,000 for wild turnip and rape respectively, but that there is a small risk of much lower or slightly higher numbers of pollen grains. Figure 23 shows the distribution used for rape flowers, but by substituting the appropriate numbers on the axis, the same distribution could be used for wild turnip.

Figure 23. Custom distribution of pollen grain number for a rape flower

Based on the data from Chapters 5 and 6 the pollen production of interspecific hybrids (pihf) is likely to be less than that of either parent but is set at the mean of the two parent's pollen production. A custom distribution similar to Figure 23 is used for the distribution (shown in Appendix 2).

*Proportion of interspecific hybrid pollen that is viable and fecund (ipg)*

The pollen of wild turnip and rape was shown to be close to fully fertile and able to germinate *in vitro*, so the parent species were assumed to have 100% fertility and
germination in the model. Pollen from the interspecific hybrids was shown in Chapter 6 to have substantially reduced fertility with <20% pollen germination, so the number of interspecific hybrid pollen grains was multiplied by 0.2 to reflect the low germination potential and is set with a uniform range of probability with minimum=0.15 and the maximum=0.25.

Seeds set per interspecific hybrid flower (if2s(wp), if2s(rp) and if2s(ip))

Female fertility is low in the interspecific hybrids (Chapter 6), where seed set was determined to be very low, even when highly fertile pollen from the parent species was used. The seed set data from the hand pollination experiments of Chapter 6 was used to develop a discrete distribution of risk. Each source of pollen is entered into the model separately, thereby representing demonstrated risk of backcross and second generation interspecific hybrid formation. The values used are presented in Table 10.

Table 10: Values used for likelihood of interspecific hybrid seed formation per silique

<table>
<thead>
<tr>
<th>Number of seeds in each silique</th>
<th>Interspecific hybrid flowers pollinated with wild turnip pollen</th>
<th>Interspecific hybrid flowers pollinated with rape pollen</th>
<th>Interspecific hybrid flowers pollinated with interspecific hybrid pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86</td>
<td>80</td>
<td>82</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
7.2.2 Calculated variables for modeling gene introgression

Frequency of successful pollinations by wild turnip (tp:p)

The frequency of successful interspecific pollinations by rape on wild turnip plants is set, with normal uncertainty (σ=0.01). The original setting is for 0.01, which was the mode of the proportion of successful interspecific pollination in the field (Chapter 3). The successful pollination of wild turnip by wild turnip is calculated from this, as all other pollinations of wild turnip maternal plants are assumed to be intraspecific. Therefore:

\[ tp:p = 1 - rp:p \]

Siliques per plant (qp)

The number of siliques per plant was assumed to be the same for wild turnip and rape, an observation based on five years of experimental work with the two species in the New Zealand environment (Chapter 3). It is a calculated value based on the number of flowers on each plant. The number of flowers used in the base model is 245, with a normal distribution (σ=1). The flower number is not the same as the silique set and so the flower number was multiplied by 0.5 to reflect the proportion of siliques that develop from flowers (Habekotte, 1993). There are fewer flowers per plant on the interspecific hybrids and the seed set in the second generation is calculated using the lower value.

Interspecific hybrid seeds formed in the first generation (i1s)

The number of ih (interspecific hybrid) seeds formed in the first year of the model system is calculated by the number of wild turnip plants in the first year multiplied by the number of siliques per plant multiplied by the proportion of wild turnip seed fathered with rape pollen:

\[ i1s = w1n*s*qp*rp:p \]
*Interspecific hybrids surviving to flowering and seed set in the second season (i2n)*

The number of interspecific hybrids which flower in the second season is calculated by the number of interspecific hybrid seeds multiplied by the proportion that successfully germinate and multiplied again by the proportion that survive juvenility to flowering:

\[ i_{2n} = i_1s \times se \times ss \]

*Pollen production of plants in the second generation (w2#pg, r2#pg and i2#pg)*

The number of pollen grains produced by the two species and the interspecific hybrids in the second season is calculated as the number of plants multiplied by the number of flowers for each plant type and multiplied by the amount of pollen per flower, i.e:

\[ w_{2pg} = w_2n \times wf \times wfp : f \text{ for wild turnip,} \]
\[ r_{2pg} = r_2n \times rf \times rfp : f \text{ for rape} \]
\[ i_{2pg} = i_2n \times if \times ipf : f \times ipg \text{ for interspecific hybrids, which includes a factor reflecting the interspecific hybrid pollen's lower fertility.} \]

The proportion of each pollen type of the total pollen population is then calculated as below:

\[ w_{2p} : p = w_{2pg} / (w_{2pg} + r_{2pg} + i_{2pg}) \]
\[ r_{2p} : p = r_{2pg} / (w_{2pg} + r_{2pg} + i_{2pg}) \]
\[ i_{2p} : p = i_{2pg} / (w_{2pg} + r_{2pg} + i_{2pg}) \]

*The number of backcross and F2 seeds produced in the second season with an introgressed gene (i2st(wp), i2st(rp) and i2st(ip))*

The number of seeds produced was calculated as the number of plants multiplied by the siliques per plant multiplied by the seed set and multiplied by the Mendelian inheritance factor:
\[ i2st(wp) = (i2n*qp*w2p:p*if2s(wp))*0.5 \]
\[ i2st(rp) = (i2n*qp*r2p:p*if2s(rp))*0.5 \]
\[ i2st(ip) = (i2n*qp*i2p:p*if2s(ip))*0.5 \]

Assuming Mendelian inheritance of an introgressed gene, 50% of the backcross progeny (to both wild turnip and rape) will inherit the transgene, as will 75% of the ih2 generation. Skewed segregation is expected in such interspecific hybrids, and the data available in Chapter 6 shows the interspecific hybrids has a greater proportion of herbicide susceptible progeny than expected. All the progeny calculations include a factor wherein they are multiplied by 0.5 to give the number of plants carrying the gene of interest.

The number of plants containing an introgressed gene of interest that survive to flowering

For the purposes of this model, it is assumed that all the backcross and ih2 hybrid progeny have a similar pattern of dormancy, germination and seedling survival to the first generation interspecific hybrids. Therefore, the i2st(wp), i2st(rp) and i2st(ip) results are multiplied by sg and ss factors to give an estimate of the number of flowering plants with the transgene in the third season:

\[ i3pt(wp) = i2st(wp)*se*ss \]
\[ i3pt(rp) = i2st(rp)*se*ss \]
\[ i3pt(ip) = i2st(ip)*se*ss \]

Seed set and fertility of these plants is unknown, and this model ends at this generation. These three figures are the outputs of the model.

7.2.3 Scenarios modeled

Five scenarios were modeled. The inputs changed in each scenario are summarized below (Table 11). Only the mean is shown, the uncertainty distribution type was not changed (Table 9), though the values for maximum and minimum were changed as appropriate for the new mean.
<table>
<thead>
<tr>
<th>Input Changed</th>
<th>W1n</th>
<th>r2n</th>
<th>rp:p</th>
<th>wf</th>
<th>Rf</th>
<th>if</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scenario One</strong></td>
<td>10</td>
<td>5</td>
<td>0.01</td>
<td>245</td>
<td>245</td>
<td>125</td>
</tr>
<tr>
<td>Scenario Two</td>
<td>10</td>
<td>5</td>
<td>0.1*</td>
<td>245</td>
<td>245</td>
<td>125</td>
</tr>
<tr>
<td>Scenario Three</td>
<td>100*</td>
<td>5</td>
<td>0.01</td>
<td>245</td>
<td>245</td>
<td>125</td>
</tr>
<tr>
<td>Scenario Four</td>
<td>10</td>
<td>200*</td>
<td>0.01</td>
<td>245</td>
<td>245</td>
<td>125</td>
</tr>
<tr>
<td>Scenario Five</td>
<td>10</td>
<td>5</td>
<td>0.01</td>
<td>400*</td>
<td>400*</td>
<td>205*</td>
</tr>
</tbody>
</table>

* Aspects changed in scenarios

The second scenario modeled the result of a greater number of successful pollinations by rape on wild turnip maternal plants.

The third scenario modeled the initial wild turnip population and the population in the second year set at 100 individuals.

In the fourth scenario the number of rape plants in the second generation was changed to 200, to reflect the possibility a different but equally close paddock was planted with rape. Although a paddock of rape contains a substantially larger number of individuals, only a small number of them would be able to contribute pollen to a ruderal wild turnip population (Lavigne *et al.*, 1998).

Colbach *et al* (2001) reports the figure of approximately 400 flowers per plant. The model was also run using this number for the rape and wild turnip, and the interspecific hybrids were estimated to have approximately half that number of flowers (200) in scenario 5.

### 7.3 Modeling transgene introgression in brassica populations

Five scenarios were examined using 10,000 simulations of the stochastic predictive model. The scenarios were chosen to reflect likely variation in the
population sizes, the successful ratio of interspecific pollinations and recorded variation in the number of flowers present on each plant.

To examine the importance of the size of the initial wild turnip population three scenarios were tested, and the effects of the change on the elasticity of the elements contributing to the model outputs were examined. The first scenario is illustrated using an example based on the New Zealand context with a population of 10 wild turnip plants occurring near a rape crop. This wild turnip population size was based on recent survey of the abundance of *Brassica* taxa in New Zealand (Heenan *et al.*, 2004). The effect of more successful pollinations of wild turnip by rape in the first generation is examined in scenario two by changing the proportion of successful rape pollinations on wild turnip from 0.01 to 0.10. The model is run a third time with a wild turnip population size of 100. This is not an unrealistic scenario since large wild turnip populations can occur in Canterbury, New Zealand, especially after soil disturbance (Figure 21). A fourth scenario examined the effect of a larger rape population occurring in the second season, possibly due to another farmer planting forage rape in a neighboring field, and is examined with a rape pollen donor population of 200 in the second season. Although a rape field would contain many more than 200 plants, only a small number of the plants are likely to contribute pollen to a ruderal brassica population (Lavigne *et al.*, 1998). There is some variation in the literature describing the morphology of rape and in particular the number of flowers borne on each plant. The fifth scenario examines the effect on the number of interspecific hybrid progeny when the number of flowers per plant is doubled.

All numbers in this section are from Table 12 unless otherwise noted in the text.
Table 12. Summary of results from model of gene introgression from rape to wild turnip under five different scenarios.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Minimum</th>
<th>Mean</th>
<th>Maximum</th>
<th>5%</th>
<th>95%</th>
<th>Model sensitive to</th>
<th>Regression value</th>
<th>Second sensitive to</th>
<th>Regression value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scenario 1</strong></td>
<td>Most likely scenario</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild turnip</td>
<td>0</td>
<td>9</td>
<td>401</td>
<td>0</td>
<td>59</td>
<td>if2s(wp)</td>
<td>0.773</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rape</td>
<td>0</td>
<td>11</td>
<td>250</td>
<td>0</td>
<td>65</td>
<td>if2s(rp)</td>
<td>0.779</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>0</td>
<td>1</td>
<td>681</td>
<td>0</td>
<td>6</td>
<td>if2s(ip)</td>
<td>0.571</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scenario 2</strong></td>
<td>Higher proportion of interspecific hybrid progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild turnip</td>
<td>0</td>
<td>48</td>
<td>1825</td>
<td>0</td>
<td>293</td>
<td>if2s(wp)</td>
<td>0.920</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rape</td>
<td>0</td>
<td>57</td>
<td>1029</td>
<td>0</td>
<td>303</td>
<td>if2s(rp)</td>
<td>0.922</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>0</td>
<td>39</td>
<td>5178</td>
<td>0</td>
<td>173</td>
<td>if2s(ip)</td>
<td>0.891</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scenario 3</strong></td>
<td>Large population of wild turnips</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild turnip</td>
<td>0</td>
<td>156</td>
<td>7402</td>
<td>0</td>
<td>1031</td>
<td>if2s(wp)</td>
<td>0.803</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rape</td>
<td>0</td>
<td>19</td>
<td>782</td>
<td>0</td>
<td>111</td>
<td>if2s(rp)</td>
<td>0.742</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>0</td>
<td>23</td>
<td>6583</td>
<td>0</td>
<td>99</td>
<td>if2s(ip)</td>
<td>0.602</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scenario 4</strong></td>
<td>Large population of ruderal rape</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild turnip</td>
<td>0</td>
<td>0.5</td>
<td>41</td>
<td>0</td>
<td>3</td>
<td>if2s(wp)</td>
<td>0.663</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rape</td>
<td>0</td>
<td>23</td>
<td>1131</td>
<td>0</td>
<td>144</td>
<td>if2s(rp)</td>
<td>0.756</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>0</td>
<td>0.09</td>
<td>103</td>
<td>0</td>
<td>0.3</td>
<td>if2s(ip)</td>
<td>0.341</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scenario 5</strong></td>
<td>Larger numbers of flowers on all plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild turnip</td>
<td>0</td>
<td>23</td>
<td>1082</td>
<td>0</td>
<td>148</td>
<td>if2s(wp)</td>
<td>0.786</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rape</td>
<td>0</td>
<td>27</td>
<td>883</td>
<td>0</td>
<td>163</td>
<td>if2s(rp)</td>
<td>0.767</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>0</td>
<td>6</td>
<td>2620</td>
<td>0</td>
<td>23</td>
<td>if2s(ip)</td>
<td>0.516</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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7.3.1 Scenario One

This scenario is based on the most realistic combination of plant population size and individual plant morphology based predominantly on New Zealand data. Turnips are likely to have a population of about 10 plants, and the ruderal rape population is likely to be small (in this case 5 plants). The ratio of successful rape pollinations used (rp:p=0.01) is based on the field experiments performed during this thesis (Chapter 3). The number of flowers per plant used in this scenario is 240 (Lefol et al., 1996).

A: Wild turnip backcrosses

The majority (>80%) of the simulations resulted in no third generation wild turnip backcross plants becoming established. The maximum number of plants produced by simulations (401) is much greater than the 95\text{th} percentile of the distribution (59), showing the rare possibility of larger numbers. This result is also shown graphically (Figure 24).

![Figure 24. The cumulative distribution of progeny of interspecific hybrids backcrossed to wild turnip (I3pt(wp), Scenario One](image)
The most influential input parameter on the results of the model was seed set on interspecific hybrid flowers pollinated with wild turnip pollen (if2s(wp)) with a value of 0.773. The proportion of interspecific hybrid plants produced by wild turnip plants is the second most influential input parameter with the much smaller value of 0.191. Tornado diagrams are generated in @risk to give a graphical interpretation to these figures (Figure 25).

Figure 25. Tornado diagram showing most influential parameter on output ‘interspecific hybrid progeny backcrossed to wild turnip’.
B: Rape backcrosses

The majority (>75%) of the simulations resulted in no third generation rape backcross plants becoming established. The maximum number of plants produced by simulations (250) is much greater than the 95th percentile of the distribution (65), showing the rare possibility of large numbers. The cumulative distribution is flatter for this output than for wild turnip backcrosses.

The most influential input parameter on the results of the model was seed set on interspecific hybrid flowers pollinated with rape pollen (if2s(rp)) with a value of 0.779. The proportion of interspecific hybrid plants produced by wild turnip plants in the first generation (rp:p) is the second most influential input parameter again.

C: Second generation interspecific hybrids

The majority (>80%) of the simulations resulted in no third generation interspecific hybrid plants becoming established. The maximum number of plants produced by simulations (681) is much greater than the 95th percentile of the distribution (6), showing the extremely rare possibility of large numbers. The cumulative distribution is steeper for this output than for wild turnip backcrosses. The mean (1) is less than half the mean number of backcross plants generated by the model, and a singular plant is so vulnerable that it could be regarded as a zero.

The most influential input parameter on the results of the model was seed set on interspecific hybrid flowers pollinated with interspecific hybrid pollen (if2s(ip)) with a value of 0.571. Similar to both backcross situations, the proportion of interspecific hybrid plants produced by wild turnip plants in the first generation (rp:p) is the second most influential input parameter.

7.3.2 Scenario Two

This scenario is based on there being a greater proportion of interspecific hybrid seed produced by the wild turnip plants in the first generation. Other researchers have found up to 90% of wild turnip progeny to be interspecific hybrids, depending on the degree of isolation of wild turnip plants from
intraspecific pollen (Jørgensen et al., 1996). At no time during this thesis did the proportion of interspecific hybrids in the seed of wild turnip plants exceed 7%, a value that was achieved using a ratio of 1:1 rape: wild turnip and optimum growing conditions (Chapter 4). The value of 10% proportion of interspecific hybrids in the seed of wild turnip plants is considered to be highly unlikely in normal conditions, but was included to show the effect of changes to this input parameter.

A: Wild turnip backcrosses

The mean and the maximum number of wild turnip backcrosses is larger than in scenario 1, but the mean (48) is still much smaller than the maximum indicating most simulations returned low numbers, and the majority of simulations are still zero. Although seed set on interspecific hybrids is still the most influential factor changing simulation outcomes, the second most important in this scenario is seed set on the wild turnips in the first generation.

B: Rape backcrosses

Again, in this scenario there is a greater likelihood of the establishment of rape backcross plants in the environment in scenario 2 compared to scenario 1, but large numbers of rape backcross plants are very unlikely. Establishment of rape backcross plants is very sensitive to the interspecific hybrid seed set.

C: Second generation interspecific hybrids

The majority (80%) of the simulations resulted in no third generation interspecific plants becoming established. The mean was 39, the 95th percentile 173 and the maximum 5178, showing the very steep cumulative distribution curve and very low change of large populations establishing. However there is a tiny chance of very large populations of second generation interspecific hybrids establishing, and the seed set on interspecific hybrids is the controlling factor with 0.891 proportion of the variation due to this factor.
Summary

There is more chance of gene introgression if there are larger proportions of progeny in the first generation receiving the gene of interest, however there is still a very high likelihood that the gene would not introgress. Too many of the simulations result in no progeny being established in the third generation.

7.3.3 Scenario Three

Large population of wild turnips can occur in Canterbury on disturbed ground, especially in areas with disturbed ground. By changing the input value \( w1n \) to 100, the consequences of a larger than normal population of wild turnip can be modeled.

A: Wild turnip backcrosses

The majority (>80%) of the simulations resulted in no third generation wild turnip backcross plants becoming established. There is a very steep cumulative distribution curve, with the maximum possible number of wild turnip backcrosses being 7402, which is almost double that in scenario 1. The model is sensitive to the same factors as in scenario 1.

B: Rape backcrosses

The majority (>75%) of the simulations resulted in no third generation rape backcross plants becoming established. The maximum and mean in this scenario are three times those found in scenario 1 showing an increasing population of wild turnips enhances the chance of gene introgression from rape to wild turnip. The model is still sensitive to the same factors as in scenario 1.

C: Second generation interspecific hybrids

The majority (>80%) of the simulations resulted in no third generation interspecific hybrid plants becoming established. However this scenario showed there was a small chance of very large populations of second generation interspecific hybrids establishing, larger than those estimated in scenario 1. The same factors influenced the outcome of the model.
Summary

Increasing the number of wild turnips in the original population and in the subsequent seasons does not change the likelihood of gene introgression, as most simulations still result in no plants carrying introgressed genes becoming established in the environment, but when they do establish, large populations are possible. Outcomes of the model are strongly effected by seed set of the interspecific hybrids.

7.3.4 Scenario Four

Scenario four is intended to model a situation where either there is a large spill of rape seed very close to a wild turnip population which flowers at the same time as the wild turnip or that another field close to a wild turnip population is planted in rape and flowers during the second generation of the model.

A: Wild turnip backcrosses

The majority of the simulations resulted in no third generation wild turnip backcross plants becoming established. The maximum number of plants established in the simulations was 41, showing that having more rape in the environment reduces the likelihood of introgression of rape genes into the wild turnip genome.

B: Rape backcrosses

The majority (>75%) of the simulations resulted in no third generation rape backcross plants becoming established. With more rape in the environment there is a greater chance of rape backcrosses establishing. It is still most likely that no plants with the new gene will be established.

C: Second generation interspecific hybrids

The majority of the simulations resulted in no third generation interspecific hybrid plants becoming established. Very few of the simulations resulted in the establishment of second generation interspecific hybrids, with a maximum of 103 but a 95th percentile of 0.3. A large population of rape has reduced the likelihood of the establishment of interspecific hybrids in the field. The same
factor of interspecific hybrid establishment in the field is the most powerful influence of the model.

Summary

Increasing the size of the population of ruderal rape has decreased the likelihood of establishment of wild turnip backcross and second generation interspecific hybrids, but increased the likelihood of the establishment of rape backcross plants compared to scenario 1. The seed set of interspecific hybrid plants in the most influential factor on the outcome of the model.

7.3.5 Scenario Five

Scenario five examines the situation where there are more flowers on all the plants in the model. There is disagreement in the literature as to how many flowers are on rape plants, and no publications known to this author examine the number of flowers on a wild turnip plant. Lefol et al. (1996) reports 240 flowers per rape plant. In this scenario the changes that might occur to the model outputs are examined, if New Zealand rape had 400 flowers as reported by Colbach et al. (2001).

A: Wild turnip backcrosses

The majority of the simulations resulted in no third generation wild turnip backcross plants becoming established, while a small number resulted in the establishment of important populations. Both the mean and the maximum number of plants predicted by the model were larger than those predicted in scenario one.

B: Rape backcrosses

The majority of the simulations resulted in no third generation rape backcross plants becoming established. Where simulation did generated rape backcross plants, the numbers generated were larger than those predicted in the first scenario.
C: Second generation interspecific hybrids

The majority of the simulations resulted in no third generation interspecific hybrid plants becoming established. Although the mean number of second generation interspecific hybrid plants was 1 in scenario 1, it was 6 in scenario 5. The maxima changed from 681 in scenario 1 to 2620 in scenario five.

Summary

Changing the number of flowers on all the types of plants increased the maxima and mean of the three outputs of the model. The majority of simulations still resulted in no establishment of the new gene in the ruderal environment.

7.4 General conclusions

The ease with which the inputs can be changed in the Excel spreadsheet makes it simple to explore the effects of changes to parameters in the original scenario. Since the model is based on a simple additive system, additional parameters can be factored into the model without excessive difficulty to reflect different environments that may influence different relationships between the two species being considered. Pictorial outputs make interpretation and communication of results relatively simple. Cumulative distribution curves show the likelihood of a range of results. Tornado graphs illustrate the parameters influencing the model output.

The model would be a useful tool for regulatory authorities, who could set a limit on the acceptable number of first generation interspecific hybrids, and work backwards to limit the number of each species allowed to be planted. Alternatively, they could set the number of original plants allowed in trials or commercial plantations and predict the consequences or granting approvals. Authorities could use the regression sensitivity analysis to set the most effective controls necessary to minimize the existence of interspecific hybrids. Applicants could use the model to suggest the most practicable controls that would still minimize environmental impacts.

All scenarios resulted in most simulations returning a prediction of no establishment of the new gene in backcrossed plants of either species or
interspecific hybrids. The situation were there were large populations of wild turnip (modelled in scenario 4) were most likely to result in gene introgression. Increasing the number of flowers on plants also increased the likelihood of introgression, presumably by increasing the number of progeny and thereby improving the odds of introgression by sheer weight of numbers.

The proportions of pollinations from rape (rp:p) was not as important as was expected. Several publications have focused on quantifying this figure (Palmer, 1962, Jørgensen and Anderson, 1994, Scheffler and Dale, 1994, Wilkinson et al., 2003, Bing et al., 1991, Jenkins et al., 1999) with a range of results reflecting genotypic and experimental design variation. From this model, it appears that the most influential input parameter in the model for all scenarios is the number of seeds set on interspecific hybrid plants. Further reductions in the transmission of genetic material could include genetic engineering of the chloroplast genome (Daniell et al., 1998) apomixis, cleistogamy, chemical induction/deletion of transgenes, fruit-specific excision of transgenes and transgenic mitigation (Daniell, 2002).

Further information would improve the accuracy of the model, but in this case the intention was to determine the most sensitive parts of the rape/wild turnip life 'cycle' to enable management controls and prevent the formation of interspecific hybrids. By testing five scenarios which significantly changed inputs and finding the same parameter constantly indicated as the most influential parameter we can confidently predict the most sensitive life cycle parameter on which to apply controls. Any reduction in the female fertility of interspecific hybrids will substantially reduce gene introgression. Reduction of fertility can be achieved very simply through mowing verges after bolting of brassicas. These interspecific hybrids have reduced vitality, and may be unable to produce more flowers from an axial bud.

@risk is primarily designed to reduce the risks to commercial organizations which are contemplating new ventures. As such it has been designed to cope with a wide range of scenarios, and this flexibility allows its application to ecological scenarios. The strong emphasis on visualization of distribution risks and outputs eases the communication of complex concepts. Further
capabilities remain to be explored in the application of this program to invasion ecology.
Chapter 8: General discussion and future prospects

8.1 Overview

This thesis aimed to quantify the risk of gene introgression from rape (*Brassica napus*; AACC; 2n = 4x = 38) to wild turnip (*Brassica rapa* var. *oleifera*; AA; 2n = 2x = 20) in the New Zealand context. From previous studies around the world there are large differences in the observed ability of wild turnip and rape to produce interspecific hybrids (e.g. Scheffler and Dale, 1994; Jørgensen and Andersen, 1994), and little research on New Zealand biotypes (Palmer, 1962). A non-transgenic homozygous dominant herbicide-resistant rape and six populations of wild turnip from a wide geographical range of habitats in New Zealand were used to investigate gene introgression. The herbicide resistance trait made it possible to screen large numbers of seeds and plants.

As a basis for risk assessment for the release of transgenic rape plants, the first objective investigated the potential for hybrid formation between the herbicide-resistant rape as the pollen parent and six New Zealand populations of wild turnip as the maternal parent. Following hand pollination, 41% of pollinated stigma developed into siliques with seeds. The frequency of successful pollination varied significantly between wild turnip populations (Figure 2) and was higher on flowers from lower racemes than upper racemes (Figure 3). The wild turnip populations also differed in the number of seeds per silique (Figure 4). In all populations, fewer seeds developed in siliques from the upper raceme compared with the lower raceme (Figure 5). Over 99% of successfully germinated progeny were chlorsulfuron-resistant, with the seed germinating as chlorsulfuron-sensitive seedlings (0.7%) presumed to be matromorphic seed (Table 3). Flow cytometry established that the chlorsulfuron-resistant seedlings were triploids, thereby confirming their hybrid status (Table 4). The frequency of dormant seeds did not differ between wild turnip populations, although the upper racemes produced 6-fold more dormant seed than the lower racemes (Figure 6). Dormancy enhances the opportunities for gene introgression by increasing the opportunities for sympatry of flowering with ruderal brassica populations.
The second objective examined the ability of wild turnip to produce interspecific hybrids in the field. Field trials were established over four summer seasons and a large number of seeds collected and screened for herbicide-resistant interspecific hybrids. No wild turnip population effects were observed for the proportion of interspecific hybrids produced (Figure 7). Populations varied significantly in the total number of seeds produced per plant in the second year, but no population effects were observed in the other years (Figure 8). There was a significantly higher proportion of interspecific hybrids produced in year 1 compared with the other three years, but no population effects or population x year interactions were observed (Figure 9). There was significantly more seed produced per wild turnip plant by all populations in year 1, but no population or population x year interactions were apparent (Figure 10). A higher ratio of rape to wild turnip resulted in a higher proportion of interspecific hybrids (Figure 11) and a larger number of seeds per plant (Figure 12). Over the four years the incidence of interspecific hybridisation ranged from <0.1%-2.1%. The variation in seed production resulted in the absolute number of interspecific hybrids produced on a per plant basis ranging from 0.146 to 161.

Knowledge of pollination events that effect interspecific hybridisation and the nature of any barriers that minimise interspecific hybridisation can provide a basis for managing the incidence of gene flow between crops and wild relatives. The third objective investigated the relative importance of honeybees and wind for interspecific hybridisation between rape and wild turnip within enclosed glasshouses with either bees (Apis mellifera) or an artificial wind-source. Interspecific hybridisation frequencies from the transfer of rape pollen to wild turnip plants showed no significant differences amongst five wild turnip populations (Figure 14). Wind pollination resulted in a significantly increased frequency of interspecific hybrids (7.2%) compared to bee pollination (4.5%) (Figure 15), but also resulted in approximately half the seed production (Figure 13). Consequently the absolute number of interspecific hybrids produced on each wild turnip plant was similar for both wind and bee pollination. Since wild turnip is self incompatible and rape has approximately double the pollen load, the substantially lower frequency of hybrids from bee pollination relative to the more random wind pollination is unexpected. This suggests that bees may be
exhibiting some floral constancy when foraging for pollen/nectar on flowers of rape and wild turnip. Markedly different light reflectance patterns within the UV spectra from flowers of the two species could be the basis for this discrimination (Figure 16).

The fourth objective was divided into two parts. The first was to examine hybrid establishment in the field after open field pollination (being the trials established in objective two). The trial sites were left for differing times and under different management regimes. At all sites a large number of brassica seedlings germinated. Only 1-3 herbicide-resistant seedlings were observed at all three sites (Table 5). All of these seedlings were found to be triploid (Table 6) and none of them set seed, despite the abundant presence of other flowering brassicas in the surrounding area. The overall frequency of interspecific hybrids successfully germinating at each site was substantially lower than the frequency of interspecific hybrids seedling originally harvested at each site (Table 5). The second part of this objective was the examination of interspecific hybrid establishment following sowing of seed mixtures. Known proportions of interspecific hybrid and wild turnip seed were sown in the field, and herbicide was applied to the split split plot design on three different dates to determine the frequency at which the interspecific hybrids established. No population effects were detected (Table 7). There was a significant decline in the proportion of interspecific hybrid seedlings over the eight week period after sowing (Table 7). A correlation analysis showed that the number and the proportion of interspecific hybrid plants decreased over time.

The fifth objective was to establish the male and female fertility of the interspecific hybrids, and the segregation of herbicide resistance in progeny of interspecific hybrids. Pollen viability of the interspecific hybrids ranged from 35-41%, as opposed to the parent species which both had pollen fertility of 100% (Figure 17). Interspecific hybrid in vitro pollen germination was only 13-17% while both species had pollen germination of virtually 100% (Figure 18). There were no population differences discernable for either test. Male fertility was low. Female fertility was measured using silique set and seed number per silique. Silique set was low, with 88% of hand pollinations failing to produce a silique (Figure 19). Of the successful pollinations 79% produced one seed, 16%
produced 2 seeds, 5% produced 3 seeds and 2% (one silique) produced 13 seeds per silique. There were no population differences. Backcrossing was a more successful method of producing seed than crossing within the interspecific hybrid plants or within the interspecific hybrid populations (Figure 20). Female fertility was also low. Segregation of herbicide resistance in progeny of interspecific hybrids was regular in backcrosses but highly skewed following self pollinations with an excess of herbicide-sensitive progeny (Table 8).

The final objective was the combination of the information and data collected in all the previous work into a stochastic predictive model using the Excel add-on @risk. A model was developed in Excel and the uncertainties associated with the data were entered as distributions. Five scenarios were tested, with variations in the size of the wild turnip and rape populations, the proportion of pollinations by rape on wild turnip maternal plants and the number of flowers on the three types of plants in the model (Table 12). Cumulative distribution curves were steep, showing high numbers of progeny to be unlikely. More than 75% of the simulations produced no progeny in all scenarios. Since the aim was to examine the introgression of rape genes to wild turnip and so the model results predicting third generation interspecific hybrid plants that have backcrossed to wild turnip are the most critical. In all scenarios the most influential input parameter was the number of seeds set on interspecific hybrid plants. This factor was always valued >0.5 of the most influential parameter contributing to the model outcome. Any further reduction in the low female fertility of the interspecific hybrids would dramatically reduce introgression of genes from rape to wild turnip. This identifies a key parameter to target management practices to minimise gene introgression from rape to wild turnip, should this be considered necessary.

8.2 Avenues for further research

The basis for the infertility of the interspecific hybrids has not been characterised in this research. It is most likely to be due to the triploid chromosomal status and the subsequent breakdown in meiosis, but this is only speculative. Further work to characterise the infertility may have applications in
the reduction or control of gene 'leakage' from crops both traditionally derived and transgenic.

The ability of rape and wild turnip to form interspecific hybrids has been examined in Canada (Bing et al., 1991, 1995, 1996), the United Kingdom (Wilkinson et al., 2000, 2003) New Zealand (Palmer, 1962, Jenkins et al., 2001) and Denmark (Jørgensen et al., 1994, 1996, 1998). The Danish group has reported radically different results from any other research group in the world. In Denmark the frequency of interspecific hybrids in field situations is reported as 10-90% (Jørgensen and Andersen, 1994), while in other parts of the world reports the frequency of interspecific hybrids as 1%. Understanding the basis for this difference could reduce the perceived risk of transgenic crop release. It would be interesting to grow their wild turnip and wild turnip from around the world and compare directly and in a controlled experiment the differences in interspecific hybrid formation, morphology and gene introgression. Only minimal population differences were observed in this thesis, but populations in New Zealand have only had a maximum of 150 years of isolation from their European sources. Significant differences may exist between populations of Europe, the Americas and the Asia-Pacific region, as indicated by the variation in the formation of interspecific hybrids.

Stable introgression and expression of a foreign gene over multiple generations has not been examined in this thesis due to constraints of time. This may not be possible due to the very low levels of fertility in the interspecific hybrids (Appendix 1), but a larger trial could produce valuable information. The ability of wild turnip plants in particular to produce fertile offspring after backcrossed pollination with interspecific hybrid pollen is an important further avenue of research.

Mathematical modeling seeks to describe biological processes in the reductionistic language of mathematics. A successful model of gene introgression could be used for any species on which data exists. Application of this model to another system such as different plant species or organisms from another kingdom could demonstrate the robustness of the model system. Examination of the most influential input parameters would show different
vulnerabilities in other introgression systems. There may be a generalised pattern of appropriate control measures for the enhancement or reduction of introgression, information that would be of value to plant breeders seeking to control their intellectual property, or regulators attempting to minimise gene leakage from transgenic crops.

This thesis involved the first application of @risk to biological problems. The @risk package offers a valuable resource for modeling invasion ecology, where the study is not the introgression of genes, but the establishment of organisms in a new environment. Invasion depends in part on the availability of food sources or appropriate ecological niches in the case of plants, the speed of growth to reproductive phases, the influence of parasites and predators and successful reproduction. Modeling presents an opportunity to prioritise invasive risks and target control measures to either the most dangerous organisms, or the most vulnerable part of their life cycle.

8.3 Contributions of this thesis to the study of introgression in brassicas

This thesis has

- Established the ability of rape and New Zealand wild turnip populations to produce interspecific hybrids in the glasshouse and field
- Ascertained that there are minimal differences between different New Zealand wild turnip populations in their ability to produce interspecific hybrids
- Ascertained that wind is the most successful vector for interspecific pollination
- Established that bees exhibit floral constancy between the flowers of rape and wild turnip, and there are not a major threat for interspecific hybridisation
- Shown that interspecific hybrid seed and seedlings have reduced success in germination and establishment in the field
Quantified the reduction in the male and female fertility of the interspecific hybrids as compared to the parent species.

Demonstrated the valuable applications of @risk for predictive stochastic modeling for gene introgression and potentially other biological investigations.
Reference List


Appendix One: Reproductive success of interspecific hybrids between wild turnip (*Brassica rapa* var. *oleifera* DC) and rape (*Brassica napus* L.) in the field.²

Previous experiments outlined in Chapter 5 established that interspecific hybrids between wild turnip (*Brassica rapa* var. *oleifera* DC) and rape (*Brassica napus* L.) failed to set seed in the field. Seed production was not limited by pollen availability since there was a large number of wild turnip pollen donors present at the edge of all herbicide-treated areas. This suggests that these interspecific hybrids have very low fecundity and consequently a very low potential for acting as a bridge for gene introgression between cultivated rape and wild turnip. However, the observed reduction in fertility in the field experiments of Chapter 5 may have been caused by the herbicide treatment applied to the interspecific hybrids in the field. It was therefore important to re-examine the reproductive success of wild turnip x rape interspecific hybrids in the field without herbicide treatments.

Seed used in this study was derived from open pollination of wild turnip as the maternal parent with rape plants homozygous at a single locus for a dominant mutation conferring resistance to the herbicide chlorsulfuron. The wild turnip plants were from five New Zealand populations of a wide geographic range (Massey, Riwaka, Marshland, Greymouth, and Makarewa; Table 2). Seeds from artificial hybridisation of the homozygous herbicide-resistant rape (30a) and an isogenic herbicide-sensitive line (wt) were also used. This seed was screened in vitro for chlorsulfuron resistance to identify interspecific hybrids using the method described in Chapter 2 (Section 2.2.3). Interspecific hybrids derived from each wild turnip population were potted into individual pots of standard potting mix as described in Conner and Christey (1997). Seeds from each of the parent wild turnip populations and the herbicide-resistant and herbicide-sensitive rape lines were also sown directly into pots with the same soil. Plants

² Based on data of A. J. Conner, Lincoln University/Crop & Food Research
were grown in a greenhouse for 2 weeks until they reached the 2-3 true leaf stage, and then hardened-off in a screen house for one week before transplanting into the field.

Plants from the 13 brassica lines (five wild turnip populations, five populations of wild turnip hybridised to rape, three rape lines) were planted in the field in December 2003 at Crop & Food Research, Lincoln on a Templeton silt loam. The trial was established as six blocks, each with randomised single row of 10 plants for each line. Within each row the brassica plants were approximately 0.2m apart, with rows approximately 0.3 m apart. The trail was surrounded on two sides by trials of forage brassica plants and on the other two sides numerous wild turnip plants. The plants within the trial were observed throughout the growing season. When fully grown, the flowering plants were touching, both within and between the rows. The development of siliques was monitored and observations on the siliques with seed on each plant were recorded at the end of the growing season (April-May 2004).

All plants from the parent populations of wild turnip and rape produced numerous siliques (Table A.1). There were usually 15-30 seeds in each silique that produced seeds. In marked contrast, none of the interspecific hybrids between wild turnip and rape formed any siliques with seed (Table A.1) despite the high availability of a variety of brassica pollen in the immediate neighbourhood. Occasionally a few rare siliques did initiate development on some hybrid plants, but in all cases all seed development aborted in these siliques. This was consistent for hybrids involving all five wild turnip populations. This confirms the very low fecundity of interspecific hybrids between wild turnip and rape when grown in the field in an agricultural setting.
Table A.1. Reproductive success of wild turnip, rape and their interspecific hybrids in the field.

<table>
<thead>
<tr>
<th>Brassica population</th>
<th>Number of siliques with seeds per plant (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild turnip populations</strong></td>
<td></td>
</tr>
<tr>
<td>Massey</td>
<td>&gt;110*</td>
</tr>
<tr>
<td>Riwaka</td>
<td>&gt;120*</td>
</tr>
<tr>
<td>Marshland</td>
<td>&gt;80*</td>
</tr>
<tr>
<td>Greymouth</td>
<td>&gt;75*</td>
</tr>
<tr>
<td>Makarewa</td>
<td>&gt;145</td>
</tr>
<tr>
<td><strong>Rape populations</strong></td>
<td></td>
</tr>
<tr>
<td>30a</td>
<td>&gt;45*</td>
</tr>
<tr>
<td>wt</td>
<td>&gt;50*</td>
</tr>
<tr>
<td>wt x 30a</td>
<td>&gt;50*</td>
</tr>
<tr>
<td><strong>Interspecific hybrids</strong></td>
<td></td>
</tr>
<tr>
<td>Massey x 30a</td>
<td>0</td>
</tr>
<tr>
<td>Riwaka x 30a</td>
<td>0</td>
</tr>
<tr>
<td>Marshland x 30a</td>
<td>0</td>
</tr>
<tr>
<td>Greymouth x 30a</td>
<td>0</td>
</tr>
<tr>
<td>Makarewa x 30a</td>
<td>0</td>
</tr>
</tbody>
</table>

*The complete number of siliques with seeds was not counted on all plants. The number stated represents a minimum number for the individual plants in each population.

Reference

Appendix Two: Ancillary graphs, Chapter 7

Truncated distribution used for $rp : p$

Values in Thousandths

Distribution of pollen number for wild turnip flowers

Values in Thousands
Distribution of pollen grain number for rape flowers

Likelihood distribution for interspecific hybrid pollen numbers