The Effectiveness of Border Areas in Confining the Spread of Transgenic *Brassica napus* L. Pollen

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Beverly Kay Staniland

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BEVERLY KAY STANILAND

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

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The effectiveness of border areas in confining the spread of transgenic *Brassica napus* L. pollen.

Major Professor: Dr. P.B.E. McVetty, Department of Plant Science

As the development of transgenic *Brassica napus* L. strains moves into field trials in open agricultural settings, there is the need for pollen flow to be controlled. Current Canadian Government regulations require either large isolation zones (200 m) or 10 m wide borders of synchronously flowering non-transgenic *B. napus*.

Border areas of 15 to 30 m wide were planted around a 60 m X 30 m central plot of bromoxynil herbicide resistant transgenic *B. napus* strains for four field trials conducted in Carman and Winnipeg, Manitoba in 1994 and 1995. Seed samples were taken from the border area at 0, 2.5, 5, 10, and 15 m for four cardinal directions and additionally at 20, 25, and 30 m for two cardinal directions. These seed samples were planted in the field in 1995 and 1996 and screened for the presence of bromoxynil resistant plants (i.e. plants which were the result of an outcrossing event).

For the four trials combined, outcrossing rates varied with by year, direction and distance. Outcrossing rates averaged about 0.70% at 0 m and declined to 0.02% at 30 m.

The introduction of pollinators (leafcutter bees at the Carman site in 1994 and 1995) did not appear to significantly influence outcrossing rates.

The bromoxynil resistant plants were screened for heterozygosity of the bromoxynil resistance trait to ensure that they were the result of an outcrossing event and not the result of contamination by a pure breeding (i.e. homozygous) bromoxynil resistant plant. Two hundred and eighty putative F_1 plants were screened for heterozygosity of the bromoxynil resistance trait. Over 93% of the putative F_1 plants screened were found to be heterozygous, i.e. true outcross plants.

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1. INTRODUCTION

Transgenic plants [plants which have genetic material which has been altered in a way that does not naturally occur by mating and/or natural hybridization (Noome 1995)] have been produced to enhance pest control, improve agronomic performance, increase product quality and improve crop nutrition (Hollebone and Duke 1994). Novel herbicide resistant crops are among the many plants produced through genetic engineering. These novel herbicide resistant crops offer improved weed control by allowing growers to use new herbicide combinations and increase use of broad spectrum, environmentally friendly herbicides (Gasser and Fraley 1989). However, potential concerns also arise with the introduction of novel herbicide resistant crops. These concerns include the opportunity for the crop to become a weed or for the transgenes (genes inserted into the plant from a foreign source conferring the novel herbicide resistance trait) to be spread to related weed species (Crawley et al. 1993).

Brassica napus L. is a member of the *Brassica* family used for edible vegetable oil production. *B. napus* is primarily a self pollinating crop (up to 80% of seed resulting from self-pollination) but may also receive pollen from foreign sources (Downey et al. 1980). *B. napus* pollen is sticky and entomophilous in nature increasing the opportunity for insects to transfer pollen within and among cultivars of the *B. napus* crop (Eisikowitch 1981).

Production of transgenic plants in outdoor agricultural settings

provides the opportunity for gene movement to other plants, including related weed species and related crops. Therefore, field trial tests of transgenic plants require gene flow (i.e. pollen movement) control. Isolation distances of 200 meters or 10 meter borders of synchronously flowering non-transgenic *B. napus* are specified in Canadian Federal Government regulations to reduce the likelihood of transgene flow from transgenic *B. napus* field trials. There is very little information on the effectiveness of border areas under Canadian conditions. In fact, at the present time, published isolation distances are often a best estimate or based on observations of accidental crop matings in the field (Wrubel et al. 1992). The purpose of this study was to determine the effectiveness of non-transgenic *B. napus* border areas in confining transgenic *B. napus* pollen. Bromoxynil herbicide resistance was used as a marker for the study as it is a trait controlled by a single Mendelian dominant gene that confers a very high level of herbicide resistance to the plant.

2. LITERATURE REVIEW

2.1 *BRASSICA NAPUS*

Brassica napus L. is a member of the *Brassica* family used for edible vegetable oil production. This crop has an indeterminate flowering habit (Downey et al. 1980).

2.1.1 THE FLOWER

The yellow *B. napus* flower is a perfect flower consisting of four sepals, four petals, an inner whorl of four longer anthers, an outer whorl of two shorter anthers and a superior ovary of two united carpels and a style with a two lobed stigma (Downey et al. 1980, Eisikowitch 1981, Free 1970, Williams 1985). *B. napus* flowers produce nectar and abundant pollen (Williams 1985). Therefore, these flowers are considered to be entomophilous in nature (Williams 1984).

B. napus flowers begin opening at approximately 04:00 and are considered fully open by 08:00 to 09:00 (Downey et al. 1980, Williams 1985). Pollen is dehisced from the anther just prior to flowers being fully opened (Eisikowitch 1981, Mohr and Jay 1988, Williams 1985). The flowers begin closing at 17:00 to 18:00 and are considered fully closed by midnight (Downey et al. 1980, Williams 1985).

B. napus plants flower for approximately 14 to 21 days (Goltz 1987), with individual *B. napus* flowers open for a period of 1 to 3 days (Eisikowitch 1981, Szabo 1985, Tayo and Morgan 1975, Williams 1985,

Wrubel et al. 1992).

2.1.2 POLLINATION

B. napus is a primarily self fertile crop (up to 80% of seed results from self-pollination) but may also receive pollen from a foreign source (Downey et al. 1980, Williams 1978, Williams et al. 1986). Studies have shown that wind, primarily through the physical movement of plants, and insects are necessary for high seed set in *B. napus* (Bilsborrow et al. 1994, Darmency and Renard 1992, Downey et al. 1980, Eisikowitch 1981, Free 1970, Olsson 1960, Sun 1937, Williams 1978, Williams et. al. 1986).

2.1.3 POLLEN

B. napus pollen is sticky and entomophilous in nature and is not dislodged from the anther by wind (Eisikowitch 1981, McVetty et al. 1989). *B. napus* pollen is approximately one third the size of corn pollen (20 X 40 µm) (Downey et al. 1980). There have been very few experiments to determine the length of time *B. napus* pollen will survive. Downey et al. (1980) stated that pollen can be stored under laboratory conditions for 4 to 5 weeks without complete loss of viability. However, it is difficult to determine how this relates to field settings. Other studies have been conducted which determine that relative humidity affects pollen longevity in *Brassica* species (Chiang 1974, Corbet and Plumridge 1985). It is thought that lower relative humidities increase the time pollen is viable (Chiang 1974). Relative humidity may also play a role in pollen release and

pollination success (Corbet and Plumridge 1985). Unfortunately, there is little known about *B. napus* pollen and the effects of the environment on the length of time it remains viable.

2.1.4 TRANSGENIC CROPS

Plant biotechnology has enhanced pest control, improved agronomic performance, increased product quality and increased the nutritional worth of the product (Hollebone and Duke 1994). There are several definitions for genetically modified organisms. The European community defines a genetically modified organism (transgenic organism) as an organism in which genetic material has been altered in a way that does not naturally occur by mating and/or natural hybridization. In the United Kingdom, an organism is genetically modified if any of the genes or genetic material in the organism: a) have been modified by means of an artificial technique: or b) are inherited or otherwise derived through any number of replications from genes or other genetic material (from any source) which were so modified (Noome 1995).

Novel herbicide resistant crops are examples of genetically modified organisms. These crops result from an alteration in the plant which results in an overproduction of the herbicide target site (i.e. if the herbicide inhibits an enzyme in the plant, the plant will produce large quantities of the enzyme in order to overcome the herbicide effect), reduced herbicide uptake, degradation of the herbicide or a lower affinity of the plant for the

herbicide (Rogers and Parkes 1995).

There are many advantages and disadvantages of novel herbicide resistant crops. These crops improve weed control by allowing growers to use new herbicide combinations, and increase use of broad spectrum, high potency, environmentally friendly herbicides (Gasser and Fraley 1989, Goodman 1987, Marshall 1995, Messéan 1996). They also allow for the implementation of new weed management strategies such as the shift from pre-emergent herbicide to post emergent herbicides to manage weed problems in the field (Rogers and Parkes 1995). Also these products may place a different selection pressure on existing weeds (Dale et al. 1993a, Gasser and Fraley 1989). There may also be decreased costs to the farmer (Goodman 1987, Gasser and Fraley 1989).

Some of the concerns with novel herbicide resistant crops include the potential of the crops to become weeds of agriculture or to invade natural habitats (Crawley et al. 1993, Ellstrand and Hoffman 1990, Keeler 1989), or they may pose a direct risk to humans, and domesticated or wild animals (Crawley et al. 1993, Keeler 1989). Numerous studies have been conducted to determine if novel herbicide resistant traits do confer an advantage to the crop or to weeds through introgression (movement of the transgene into the weed population through cross pollination). Gene introgression may create new weeds or make present weeds more of a problem (Crawley et al. 1993, Dale et al. 1993a, Marshall 1995, Raybould

and Gray 1994, Wrubel et al. 1992). It has been suggested that novel herbicide resistance does not increase invasiveness of crops into natural habitats (Crawley et al. 1993, Dale 1994, Raybould and Gray 1994). Thill (1996) reports that novel herbicide resistant crops have the same weediness potential as other cultivars and there is no evidence that these crops are more fit than other cultivars. Wrubel et al. (1992) stated that if the non-transformed plant is not considered a weed, changes in one gene should not transform the plant into a weed. DeGreef et al. (1989) determined that glufosinate resistant potato had the same agronomic performance as non-transgenics. Forcella (1987) studied atrazine resistant *B. napus* and determined that there were some yield reduction, reduced germination, reduced emergence and a modification of the chloroplast proteins involved in photosynthesis. But these atrazine resistant *B. napus* crops can be grown in atrazine polluted soils and offer a management solution for problem weeds such as wild oats in high densities (Forcella 1987).

Selective herbicides work on genetic differences between weeds and crops (Miflin 1995). The move to novel herbicide resistant crops may shift the selection pressure placed on the weeds by the herbicide being used. There is some concern that these crops may cause a reliance on one chemical and the move away from integrated weed management strategies (Morrison 1993).

Concerns also arise over the stability of the transgenes. Loss of

transgenes in the crop may occur by actual physical loss, loss in expression or alterations in tissue expression patterns in transgenic plants (Jones et al. 1996).

The greatest concern with transgenic novel herbicide resistant crops is expressed by Dale (1994). He stated that the introduction of a single type of herbicide resistance into a crop poses little risk for development of more aggressive weeds. However, if several forms of herbicide resistance exist in the crop there is the opportunity for gene pyramiding to occur in the crop and in related weeds. A variety of the crop or a weed may be created which is resistant to several herbicides, making subsequent control of the plants very difficult.

2.2 GENE FLOW

Gene flow in plants can occur through pollen or seed movement (Ennos 1994, Raybould and Gray 1993). However, gene exchange is determined by the size, density and shape of donor and recipient populations, plant height, crop breeding systems, characters of the surrounding vegetation, terminal velocity of pollen and seeds, pollen and seed production, foraging behaviour of pollen and seed vectors and distance between populations (Bateman 1947a, 1947b, Keeler et al. 1996, Levin and Kerster 1974, Scheffler et al. 1993).

Two common methods for reducing gene flow between differing types of the same crop are border areas (also referred to as trap crops) and

isolation distance between crops (Ellstrand and Hoffman 1990, Kareiva et al. 1994, Keeler et al. 1996, Levin and Kerster 1974, Rogers and Parkes 1995, Wrubel et al. 1992). In insect and wind pollinated crops, complete containment of pollen is not possible (Kareiva et al. 1994). In these cases, it has been determined that pollen flow decreases rapidly with increased distance from the source but reaches a point where increased isolation distance from the source will not lower the rates of gene flow further (Bateman 1947a, 1947b, Handel 1983, Kareiva and Morris (in press)).

The introduction of transgenic *B. napus* varieties has increased the need to contain pollen flow in this crop. In Canada, transgenic, *B. napus* field trials are isolated from commercial *B. napus* production under Federal Government regulations of the Plant Products and Plant Health Directorate of Canada (Beversdorf 1991). These regulations state that there must be 10 m of synchronously flowering non-transgenic *B. napus* surrounding field trials or a 200 m isolation distance (Agriculture and Agri-Food Canada - Plant Products and Plant Health Directorate of Canada).

2.2.1 WIND POLLINATION

The ability of wind to act as a pollinating agent depends on the number of pollen grains produced, pollen size (i.e. weight), and sculptural characteristics (i.e. stickiness), flower structure and location, timing of flowering and timing of pollen release (Whitehead 1983). Although wind is capable of carrying considerable amounts of pollen, the amounts of pollen

in the air fluctuate with time of day and stage of flowering (Williams 1984). Williams (1984) stated that pollen levels in the air will be lowest during the night and early morning and maximum pollen levels in the air will occur between 11:30 and 14:30. In the case of *B. napus*, the highest levels of pollen in the air occur during the three week peak flowering period (Williams 1984).

Since air moves in large masses which are broken up by turbulence, pollen will be dispersed downwind without regard to species (Bateman 1947b). Generally, pollen is lifted into the air by turbulence and moves horizontally with the air currents (Levin and Kerster 1974). This pollen will be deposited near its source. Bilsborrow et al. (1994) determined that the amount of pollen moved by the air decreased by 50% in the first 2 m from the source. This will occur by pollen contacting a surface and generally not by gravitational pull (Levin and Kerster 1974). Therefore, the concentration of a specific type of pollen in the air decreases with increasing distance from the pollen source or plant (Levin and Kerster 1974). For example, when transgenic *B. napus* pollen is present in the air, it represents a very small fraction of the pollen *B. napus* plants are exposed to (Kareiva et al. 1994). Therefore, increased distances from the transgenic pollen source reduces a plant's chances of being pollinated by transgenic *B. napus* pollen.

Eisikowitch (1981) conducted studies using a hairdryer that simulated wind pollination in *B. napus* and found that the sticky *B. napus*

pollen remains adhered to the anther under relatively high wind velocities. This indicates that cross pollination of *B. napus* by wind is unlikely. However, it was also observed that small clouds of pollen grains were seen to burst out from the anther whenever the anthers were disturbed (Eisikowitch 1981). This would suggest that during insect pollination of *B. napus*, pollen could be released into the air allowing for potential cross pollination by wind.

Other studies indicated that wind plays a considerable role in the pollination of *B. napus* (Free and Nuttall 1968, Williams 1978, 1985, Williams et al. 1986). These scientists indicated that plant movement caused by the wind increases self pollination through physical contact between the anthers and stigma of the same flower. Williams (1978) reached this conclusion by conducting glasshouse experiments in which wind was controlled. She determined that yields of plants which were exposed to physical movement (shaking of the plants) had greater yields than those plants grown in still air. Unfortunately, wind is an unpredictable pollination agent of *B. napus* (Eisikowitch 1981) and cannot be relied upon to increase the yields of a *B. napus* crop. Most studies suggest that wind is not a significant pollination agent for *B. napus* crops.

2.2.2 POLLINATION BY INSECTS

Flowers of an oilseed rape (*B. napus*) crop are very attractive to insects (Picard-Nizou et al. 1995). Bees forage on the two nectaries at the

base of the outer stamens of the *B. napus* flowers (Meyerhoff 1958 as cited in Free and Nuttall 1968). Bees pollinate the *B. napus* flower while foraging primarily for nectar. Although honeybees collect significant amounts of *B. napus* pollen, it is rare for honeybees to actively scrabble (biting and scraping the anther to physically remove the pollen) for pollen from *B. napus* flowers (Free 1970, Free and Nuttall 1968, Mohr and Jay 1988). The effectiveness of *B. napus* flower pollination is reduced when the bees thieve nectar from the flower. It has been estimated that honeybees thieve nectar from up to 65% of the *B. napus* flowers they visit (Mohr and Jay 1988). The relatively small size of the *B. napus* flower and its nectar resources ensure that bees must fly from flower to flower in order to collect enough nectar, thus increasing the time a bee spends foraging on a plant and therefore, the chance of pollen being transferred (Mohr and Jay 1988).

The effectiveness of an insect pollinator depends to a large extent on the location of the pollen storage on the insect's body (Levin et al. 1971) and the availability of the pollen to pollinate the next flower visited by the insect. The amount of insect activity in the crop is influenced by environmental conditions (Langridge and Goodman 1982) and the stage of flowering of the crop (Free and Nuttall 1968). Bees move independently of one another carrying pollen from flower to flower (Bateman 1947b). Bees flying from a flower often visit the nearest plant where new pollen collected theoretically replaces pollen which is already on the body of the bee

(Bateman 1947b). Cresswell (1994) used a transgenic marker to determine that 91% of outcrossing due to pollination by a single insect occurred on the first four plants visited after a visit to a transgenic flower and no outcrossing was found to occur beyond the fourteenth flower visited.

Studies have been conducted to determine if bees show a directionality in their flight patterns within a field. These studies indicate that no true over all directionality exists for honeybees and bumblebees (Bateman 1947b, Levin et al. 1971, Woodell 1978). There does however, appear to be a successive flight directionality which is thought to be a general adaptation of the insect to reduce the probability of returning to the same flower on the same plant during nectar collection (Levin et al. 1971). It is thought that the direction of the wind may also play a role in flight directionality of insects such that the insects will fly upwind towards potential sources of nectar (Woodell 1978). Directionality is only a factor in plants where pollen is carried beyond the next flower visited (Levin et al. 1971). If pollen is moved beyond the next plant visited there is the opportunity for gene flow to occur over great distances. This is most likely to occur in plants with few flowers and that bloom over long periods of time which causes the insects to visit more plants to collect nectar (Levin et al. 1971).

Robertson and Cordona (1986) found that when honeybees are given the choice between faba beans and *Brassica*, they show a strong foraging

preference for the *Brassica* flowers. *B. napus* crops present a nectar source for bees well into the season, and continue to provide forage for bees even after peak flowering has occurred and the plants have reached the seed bearing stage (Free and Ferguson 1980). Numerous studies have been conducted on *B. napus* to determine the effect of pollination by honeybees. Honeybees are effective pollinators of *B. napus* which shortens the pollination period. This benefits the producer by increasing the quality of the crop through faster, more even maturing, and by making harvesting easier (Williams 1985, Williams et al. 1987). However, numerous researchers reported that there have been no demonstrated beneficial effects of insect pollination on the yield of *B. napus* (Free and Nuttall 1968, Langridge and Goodman 1982). While other researchers (Williams 1978, Williams et al. 1987) indicated that insect pollination may in fact increase the seed yield of *B. napus*. It is difficult to determine if bees are beneficial or necessary. However, in situations where cross pollination is desired such as hybrid crop development, insect vectors are necessary for crop production (McVetty et al. 1989). Insect vectors favour pollen transfer (Free 1970) and therefore, if insects do have an effect on crop pollination it is a beneficial one and not a detrimental effect.

Pollen flow in entomophilous plants is a function of pollinator flight distance and pollen deposition schedule (rate at which pollen is removed from the body by the stigmatic surface) as well as directionality of pollinator

flight (Levin et al. 1971). Plant density also plays a role in the distance pollen is dispersed, higher plant densities reduce the distance pollen will be moved (Levin et al. 1971). This is believed to occur because the pollinator will visit flowers nearest the one they are foraging and through pollen deposition on the nearest flower the pollen will not be moved as great a distance when plant densities are high.

2.2.3 *B. NAPUS* TO *B. NAPUS* CROSSING

Gene movement between cultivars of *B. napus* can greatly influence the crop's final characteristics and quality. In the case of erucic acid content, 8% contamination in a field of low erucic acid *B. napus* (i.e. canola quality) by high erucic *B. napus* types will raise the erucic acid content of the crop above the accepted 2% level (Bilsborrow et al. 1994). Bilsborrow et al. (1994) determined this using a volunteer contamination study in which high erucic contaminants were planted at levels of 0, 2, 4, 6, 8, 10% into low erucic plots. These plots were harvested and erucic acid content measured. Contamination levels of 8% high erucic plants in a low erucic plot were sufficient to raise the overall erucic acid content of the oil to greater than the acceptable 2% erucic acid level for canola quality oil. In this case, the crop can no longer be sold as an edible vegetable oil crop.

Specialty characteristics such as low erucic acid content have made it necessary to determine the level of outcrossing which can occur in *B. napus*. High levels of outcrossing may make it necessary to use a form of

pollen flow containment around specialty cultivars of *B. napus* to prevent these *B. napus* cultivars from contaminating other *B. napus* cultivars.

The outcrossing rates of *B. napus* have been determined by numerous researchers on a per plant basis. Using petal colour as a marker, Olsson (1952 as cited in Rakow and Woods 1987) determined outcrossing in two winter *B. napus* cultivars to be 34.6% on average for trials conducted in Sweden. Gowers (1981) found outcrossing to vary between 16 and 43% when seed colour was used as a marker for trials conducted in Scotland. Leckie et al. (1993) used powdery mildew resistance in rapid cycling *B. napus* to determine outcrossing rates of 46 to 89% in *B. napus* for trials conducted in the United Kingdom. Persson (1956 as cited in Rakow and Woods 1987) determined that interplant outcrossing in winter *B. napus* was 27 to 30% and 36% in summer *B. napus* for trials conducted in Sweden. Rakow and Woods (1987) used erucic acid content as a marker to determine outcrossing rates of 12.5 to 32.8% with an average of 21% on a per plant basis for trials conducted in Saskatchewan. Lewis and Woods (1991) recorded outcrossing rates of 15 to 95% for *B. napus* on a per plant basis using erucic acid content as a marker in the Peace River region of Canada. Becker et al. (1992) found that the rate of outcrossing on an individual spring type *B. napus* plant varied between 12 and 47% with highest rates on the lower portion of the plant and lowest rates at the top of the plant for trials conducted in Sweden.

B. napus outcrossing rates have also been determined on a plot to plot basis by researchers. Huhn and Rakow (1979 as cited in Rakow and Woods 1987) determined that the plot to plot outcrossing rate of winter *B. napus* was 5 to 15% for trials conducted in Germany.

Free (1970) determined that most contamination in a crop occurs within 90 m of the pollen source. He stated that it is necessary to isolate varieties or species of plants from those with which they readily cross.

It is obvious that the method of determining outcrossing, the trait used to determine the level of outcrossing and the location in which outcrossing is tested all effect the quantity of outcrossing which occurs. It is also clear that pollen moves from plant to plant in *B. napus* and methods of pollen containment are necessary if pollen movement is to be minimized.

2.2.4 *B. NAPUS* CROSSING TO WEEDS

One of the greatest concerns with novel herbicide resistant transgenic crops is the potential for gene movement into wild species. Darmency (1994) states that in order for hybridization to occur, the time of flowering must overlap between the pollen donor and the pollen receptor. Therefore, the flowering period of the weed (pollen receptor) and the transgenic *B. napus* (pollen donor) must overlap in order for transgenes from the transgenic *B. napus* to be transferred to the weed species. Numerous studies have been conducted to determine which weeds will cross, and to what extent they will cross, with *B. napus*.

The ability of *B. adpressa* to cross with *B. napus* was studied by Eber et al. (1994), Dale (1992), Kerlan et al. (1991, 1992), and Lefol et al. (1991). Eber et al. (1994) determined that *B. adpressa* could spontaneously hybridize with *B. napus* but 87% of the progeny were sterile and the remaining 13% of the progeny had 0 to 30% fertility. In another study, Lefol et al. (1991) produced hybrids between *B. adpressa* and *B. napus* by artificial hybridization of male sterile *B. napus*. In this case, 600 hybrids were produced per square meter and again, over 70% of the hybrids produced were sterile and the remaining 30% of the hybrids had less than 30% pollen fertility. In a study by Dale (1992) it was determined that the cross between *B. napus* and *B. adpressa* was unlikely to occur in nature. Furthermore, Kerlan et al. (1991) produced only sterile progeny when *B. napus* and *B. adpressa* were crossed. These studies indicate that the likelihood of crosses between *B. napus* and *B. adpressa* is very low.

Crosses between *Sinapis arvensis* and *B. napus* are unlikely to occur in natural situations (Bing et al. 1991, Dale 1992, Darmency 1994). Only Kerlan et al. (1992) were able to produce seeds from a cross between female *B. napus* and male *S. arvensis*.

Crosses between *B. juncea* and *B. napus* are rare (Bing et al. 1991, Fernandez-Serrano et al. 1991). Meng and Lu (1993) crossed female *B. napus* by male *B. juncea* and found embryo development to be slow and little to no seed production occurring.

Crosses between *B. napus* and *Raphanus raphanistrum* can occur and some seed may be produced (Darmency 1994). Kerlan et al. (1992) were able to produce small amounts of seed using *B. napus* as the male or pollen source. However, Kerlan et al. (1991) were unable to produce anything but sterile or partially fertile hybrids from reciprocal crosses of *B. napus* and *R. raphanistrum*. In a study by Eber et al. (1994) it was determined that natural hybridization is possible between *B. napus* and *R. raphanistrum* but 65.4% of the hybrids were produced from this cross were sterile and 34.6% had some fertility. Also, the hybrids produced by Darmency et al. (1995) were of low vigour and low fertility.

Bing et al. (1991) determined the crosses between *B. napus* and *B. nigra* were unlikely to occur in the wild. Kerlan et al. (1991, 1992) were able to produce hybrids only when *B. napus* was the female in the cross and these progeny were sterile or only partially fertile.

B. oleracea and *B. napus* were found to cross when either acted as the female parent (Kerlan et al. 1992). However, *B. oleracea* and *B. napus* crosses produced sterile progeny in studies conducted by Kerlan et al. (1991).

Darmency (1994) and Darmency et al. (1992) crossed male sterile *B. napus* with *Hirschfeldia incana* to produce approximately 25 hybrids/m^2. These hybrids had low pollen tube growth and low ovule fertilization. Furthermore, the F_1 hybrid seeds that were produced had low germination.

Crosses between *B. napus* and *B. rapa* (syn. *campestris*) can occur producing hybrids (Beversdorf et al. 1980, Bing et al. 1991, Gowers 1982, Jensen et al. 1992, Jorgensen and Andersen 1994, Keeler et al. 1996, Mikkelsen et al. 1996). However, Beversdorf et al. (1980) determined that the cross between *B. napus* and *B. rapa* would be rare. In contrast, Jorgensen and Andersen (1991) determined spontaneous hybridization would occur in field situations. Additionally, Bing et al. (1991) and Jensen et al. (1992) found that hybrid frequency increased when *B. napus* was the female. However, Gowers (1982) and Jorgensen and Andersen (1994) state that there needs to be selfing or backcrossing beyond the F_1 to produce viable hybrids. The viable hybrids of *B. napus* and *B. rapa* produced by Jorgensen and Andersen (1994) show *B. rapa* characteristics .

2.2.5 OUTCROSSING STUDIES IN TRANSGENIC CROPS

With the introduction of transgenic novel herbicide resistance into *B. napus*, the opportunity to measure outcrossing to non-transgenic *B. napus* has arisen. The use of dominant markers (such as novel herbicide resistance) allows for large scale field screening programs. Herbicide resistance is a rapid marker for screening on the whole plant level (Stalker et al. 1988). Also, some transgenic plants provide an easy mechanism for tracking pollen movement as they contain marker genes (Wrubel et al. 1992).

Timmons et al. (1996) used glufosinate resistance as a marker to

determine outcrossing in *B. napus* in Scotland. Through the use of pollen traps, transgenic pollen was measured 0, 100 and 300 m as well as 1.5 and 2.5 km from the transgenic pollen source (3 to 10 ha fields of transgenic *B. napus*). They determined that transgenic pollen density decreased with distance from the transgenic pollen source. They further studied the percentage of hybrids present at distances from the transgenic pollen source. Outcrossing or frequency of herbicide resistant hybrids at 0 m was found to be 6.3%, at 100 m it was 0.5% and at 360 m it was 3.7% (NOTE: only 137 plants screened at 360 m from the pollen source). PCR and RAPD analysis were used to determine if the potential outcross plants (plants showing resistance to glufosinate) carried the gene for glufosinate resistance.

In another study conducted by Morris et al. (1994) in California and Georgia, USA, kanamycin resistance in transgenic *B. napus* was used as a marker to determine outcrossing. These researchers used an existing Calgene, Inc. trial to determine the effectiveness of isolation zones of 4 or 8 m, trap beds and the combination of isolation zones and trap beds in containing transgenic *B. napus* pollen. The trap crop beds were sampled at distances of 0, 0.3, 0.6, 3 and 4.6 m into the trap bed. Samples were screened using a seed bioassay method in which the seeds were germinated on a kanamycin rich media and scored 7 days later for green growth (resistant) or yellow arrested (susceptible) states.

For the Morris et al. 1994 study, gene escape or pollen flow differed between sites. Short isolation distances of 4 and 8 m increased pollen movement beyond the isolation distance. In the continuous beds there was a rapid decline in the number of outcrossing hybrids for the first 0.5 m of trap bed with a slower decline over greater distances. Morris et al. (1994) determined that short isolation distances allowed increased pollen movement beyond the isolation zone. Combinations of short isolation zones and trap beds were also less effective than continuous trap beds in containing pollen when the area between transgenic *B. napus* pollen source and other *B. napus* crops is limited.

Wilkinson et al. (1995) report on a study conducted at the Scottish Research Institute in which gene flow was measured between adjacent fields of spring and autumn sown *B. napus*. Samples were taken from the autumn *B. napus* and were sown late the next summer to avoid vernalization. Plants which flowered were considered hybrids. Further RAPD analysis was conducted on the putative hybrids to ensure they carried the diagnostic marker of the spring *B. napus* cultivar. Again in this study, hybrid frequency declined with distance from the pollen source and remained stable beyond 32 m from the pollen source at 0.03 to 0.05%. In a related study reported by Wilkinson et al. (1995), emasculated plants were used to determine the effect of distance on gene flow. One set of plants were pre-pollinated to represent heavy pollen competition. Hybrids

occurred in this population 100 m from the source. In another population, emasculated plants were used to represent a population of male sterile plants, hybrids were found in this population 1.5 km from the pollen source.

In a *B. napus* outcrossing trial in England reported by Dale et al. (1993b) and Scheffler et al. (1993), glufosinate ammonium resistance was used as a marker. A 9 m circular plot of transgenic glufosinate ammonium resistant *B. napus* was surrounded by a border of non-transgenic *B. napus*. Within the 9 m transgenic plot there was a central circular 1 m plot of non-transgenic *B. napus*. The border was sampled eight times at distances of 1, 3, 6, 12, 24, 36, and 47, m and four times from the corners at 70 m from the transgenic *B. napus*. This study determined that levels of outcrossing decreased rapidly to 12 m and then remained at low levels to 70 m from the pollen source. Outcrossing in the central plot was 4.8%, 1.6% at 1 m, 0.016% at 12 m and 0.00034% by 47 m from the pollen source. Outcrossing was determined by planting harvested seeds, spraying the seedlings at the one to four leaf stage and living plants were classified as resistant. Spraying occurred two times. A southern blot analysis was used to confirm the heterozygosity (results of outcross events) of the plants surviving the herbicide treatments.

2.3 BROMOXYNIL HERBICIDE

Bromoxynil herbicide is a surface contact herbicide which inhibits

photosynthesis in broadleaf plants. The inhibition of photosynthesis occurs during photosystem II where electron transport is prevented (Freyssinet et al. 1996, Stalker et al. 1996). This inhibition of photosynthesis prevents the plant from converting sunlight into plant energy and the plant usually dies in 3 days or less.

Bromoxynil herbicide is a relatively short lived chemical with a half life of approximately two weeks in the soil. The rapid decomposition of bromoxynil is due to the ability of microorganisms in the soil to detoxify the herbicide (Freyssinet et al. 1996). A specific gene (often referred to as the BXN gene) produces an enzyme called nitrilase which converts bromoxynil into non-phytotoxic polar compounds (the nature of these polar compounds is still under investigation) (Eberlein et al. 1994, Freyssinet et al. 1996, Stalker et al. 1996). This BXN gene has been isolated from the microorganism *Klebsiella ozaenae* found in bromoxynil treated soils (Stalker and McBride 1987) and has been cloned and inserted into several crop plants such as tobacco, cotton, potato and oilseed rape (Eberlein et al. (1994), Freyssinet et al. 1996, Stalker et al. 1996).

Bromoxynil resistance in plants is a trait controlled by a single dominant Mendelian gene (Stalker et al. 1988). Similar to the microorganisms, bromoxynil resistant plants are able to rapidly metabolize bromoxynil to non-phytotoxic polar compounds within the plant at all stages of growth. A bromoxynil resistant plant is capable of metabolizing

up to 4800 g ai/ha of bromoxynil (16 X field rate) with no injury to the plant (Freyssinet et al. 1996, Stalker et al. 1996).

Studies indicate that bromoxynil herbicide is neither translocated nor concentrated within a plant reducing the risk of herbicide residues in the crop and the products produced from the crop (Stalker et al. 1988). Freyssinet et al. (1996) studied the effect of the BXN gene and the bromoxynil herbicide treatment on spring and winter oilseed rape varieties. They found that neither the BXN gene nor the herbicide treatment caused a yield reduction in the plant. Incorporating the bromoxynil resistance gene into *B. napus* crops will provide an additional broadleaf weed control option in these crops. The above studies indicate that the BXN gene and bromoxynil herbicide application should not reduce crop yield and could potentially reduce chemical input costs for the growers.

3. MATERIALS AND METHODS

3.1 OUTCROSSING RATE FIELD TRIALS

Transgenic *B. napus* outcrossing rate field trials were established in 1994 and 1995 at the University of Manitoba Research Farm at Carman and on the University of Manitoba campus at Winnipeg. In both years, the outcrossing rate trials consisted of a central bromoxynil resistant transgenic *B. napus* plot and a synchronous flowering border of bromoxynil susceptible non-transgenic *B. napus* plants. The bromoxynil resistance gene is a single nuclear dominant gene. At each location the central bromoxynil resistant transgenic *B. napus* plot was approximately 30 m by 60 m. The non-transgenic border was 15 m and 30 m on the sides and ends, respectively, of the trial. The border consisted of a 50:50 mixture of the *B. napus* cultivars, Legend and Cyclone.

The central plot and border were seeded with a small plot cone seeder at a rate of 8 kg/ha. An insecticide (Carbofuran) was banded with the seed at a rate of 12 kg ai/ha. A 1.5 m rotovated strip existed between the central plot and the border on all sides. A diagrammatic representation of an outcrossing rate field trial is presented in Figure 1.

The Carman trial was seeded on May 30, 1994 and on May 24, 1995, while the Winnipeg trial was seeded on May 18, 1994 and on May 29, 1995.

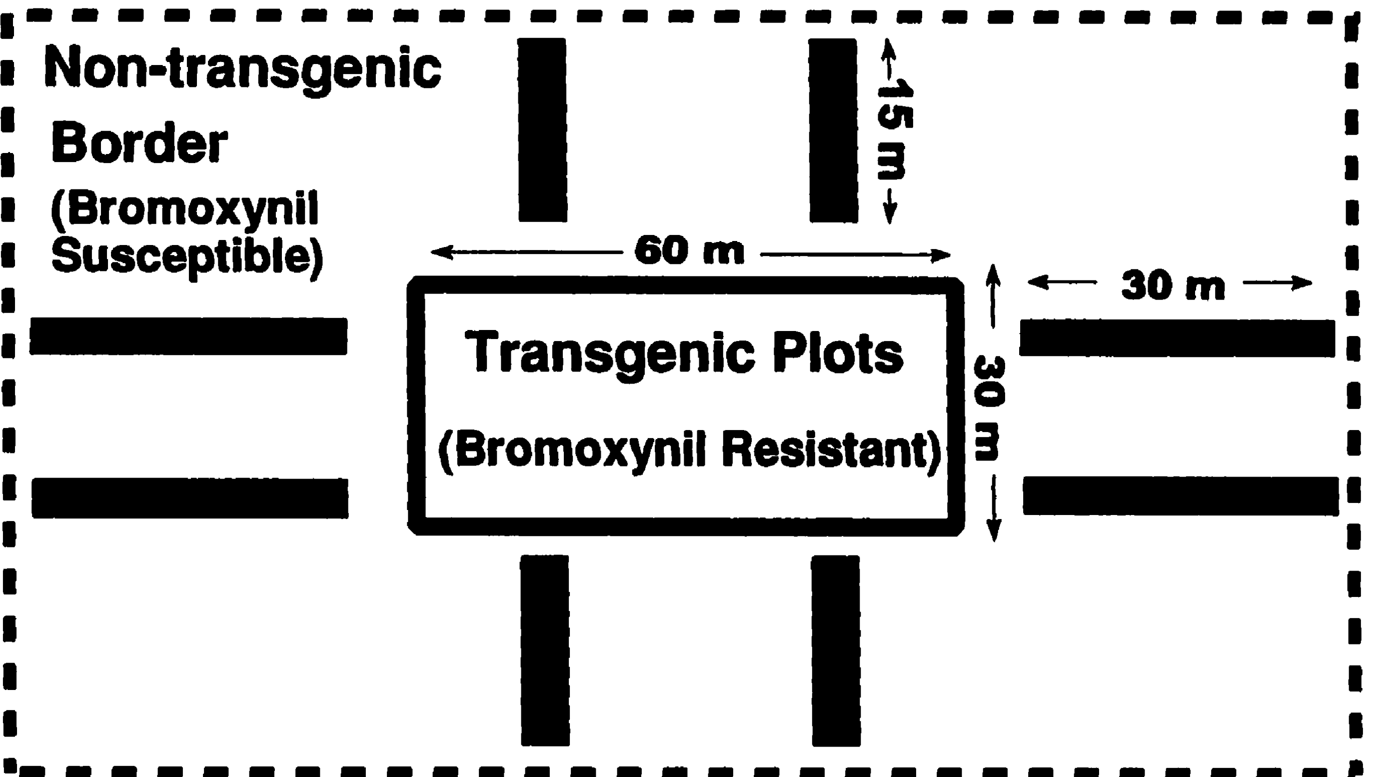

Figure 1. Diagrammatic scheme of an outcrossing field trial.

In 1994 and 1995, the Carman trials had leaf cutter bees (*Megachila rotunda*) introduced at early flowering and removed at the end of flowering. In 1994, fifty thousand bees were introduced into the trial on July 17 and removed on August 16. In 1995, sixty-five thousand bees were introduced into the trial on July 10 and removed on August 11. Two leaf cutter bee shelters were placed within the trial approximately 15 m from each edge with approximately 30 m between the two shelters. Therefore, the shelters were approximately equidistant (15 m) from three edges of the trial.

At the end of flowering the borders were mowed, leaving two 5 m wide strips of the border remaining standing in each of the four cardinal directions.

Seed samples (approximately 1 to 2 kg in size) were taken at maturity, from border areas on the sides and ends at distances of 0, 2.5, 5, 10, and 15 m, and from border areas on the ends of the trials, at additional distances of 20, 25, and 30 m. The Carman trials were harvested on August 25, 1994 and August 15, 1995, while the Winnipeg trials were harvested on August 24, 1994 and August 24, 1995.

3.2 SCREENING TRIALS

Screening trials to determine rates of outcrossing from the outcrossing rate trials were conducted in the field in 1995 and 1996 at the University of Manitoba campus at Winnipeg.

The screening trials were planted on May 26, 1995 and on June 12,

1996. In both years, a small plot cone seeder was used to plant the trials at approximately 10 kg/ha. An insecticide (Carbofuran) was banded with the seed at a rate of 12 kg ai/ha. In 1995, the plots were 28 m long and 1.2 m wide and in 1996 the plots were 30 m long and 1.2 m wide. In both cases, the plots consisted of six rows 20 cm apart. Field preparation for the screening trials consisted of a postplant pre-emergent application of glyphosate (Round-Up) herbicide on May 26, 1995 at a rate of 2.5 L/ha. In 1996, the field was cultivated on June 10 and harrowed on June 11.

The 1995 screening trial consisted of samples from the 0, 2.5, 5, 10, 15 and 30 m distances from the first replicate for all directions of both outcrossing trials. Two hundred and thirty one plots were planted in an area of approximately 1.0 ha resulting in 2 700 000 seeds being planted to be screened for the bromoxynil resistance trait.

The 1996 screening trial consisted of the remaining 1994 outcrossing trials samples and all the 1995 outcrossing trials samples (for sites, directions, distances, and replicates). Four hundred and fifty six plots were planted in an area of approximately 2.0 ha resulting in approximately 4 300 000 seeds being planted to be screened for the bromoxynil resistance trait.

Emergence began on May 30, 1995 and June 16, 1996 and emergence counts were conducted from June 6 to 13, 1995 and on June 21, 1996.

In 1995, the number of seedlings per plot was highly variable and after over three hundred 3 row/1 meter counts the number of seedlings was found to be 156.15 per m^2 with a standard deviation of 54.05. To reduce the variability the plots were visually assessed as low, medium or high emergence plots. The high emergence plots had an average density of 223.13 plants per m^2 with a standard deviation of 33.6. Medium emergence plots had an average density of 157.49 plants per m^2 with a standard deviation of 13.91. Low emergence plots had an average density of 101.80 plants per m^2 with a standard deviation of 24.16. Therefore, the high emergence plots were assigned an average number of seedlings of 7000, the medium emergence plots approximately 5200 seedlings and low emergence plots approximately 3500 seedlings per plot.

In 1996, the average number of plants per m^2 was 162.69 with a standard deviation of 33.1, producing an average of 5850 seedlings per plot. The total number of seedlings screened in the 1995 and 1996 screening trials are presented in Table 1.

The 1995 and 1996 screening trials were sprayed twice with bromoxynil herbicide, at 560 to 800 g ai/ha. The first application took place on June 19, 1995 and on June 26, 1996. A second spraying was performed to confirm that the plants surviving the first spraying were truly resistant and not the result of a spray miss (i.e. escapes). The second spraying took place on June 23, 1995 and on July 9, 1996.

The number of bromoxynil resistant plants was assessed after the first spray on June 21 and 22, 1995 and on June 28, 1996 and again after the second spray on June 25, 1995 and on July 11, 1996. In both years, the number of bromoxynil resistant plants was then compared to the total number of seedlings per plot to calculate percent resistant individuals or outcrossing rate per plot.

Table 1. Plots seeded, seeds planted and seedlings screened (combined over directions)

Year and Distance (m)	Carman			Winnipeg		
	Plots (no.)	Seeds (no.)	Seedlings (no.)	Plots (no.)	Seeds (no.)	Seedlings (no.)
1994						
0	24	229 000	160 000	24	229 000	160 000
2.5	18	179 000	125 000	24	229 000	160 000
5	24	229 000	151 000	24	229 000	142 000
10	36	359 000	227 000	36	359 000	246 000
15	27	284 000	172 000	48	488 000	274 000
20	12	100 000	70 000	12	100 000	70 000
25	15	155 000	82 000	12	100 000	70 000
30	9	97 000	44 000	45	471 000	251 000
Total	165	1 632 000	1 031 000	225	2 205 000	1 373 000
1995						
0	24	200 000	140 000	24	200 000	140 000
2.5	24	200 000	140 000	24	200 000	140 000
5	24	200 000	140 000	24	200 000	140 000
10	24	200 000	140 000	21	174 000	123 000
15	21	174 000	123 000	24	200 000	140 000
20	12	100 000	70 000	12	100 000	70 000
25	12	100 000	70 000	12	100 000	70 000
30	12	100 000	70 000	6	50 000	35 000
Total	153	1 274 000	893 000	147	1 224 000	858 0000
1994 and 1995						
Total	318	2 906 000	1 924 000	372	3 429 000	2 231 000
1994 and 1995, Carman and Winnipeg						
Grand Total	690	6 335 000	4 155 000			

3.3 GREENHOUSE CONFIRMATION TRIALS

In 1995, 28 plants and in 1996, 259 plants that were judged to be resistant, after the two applications of bromoxynil in the field, were selected at random, transplanted into pots and grown to maturity in the greenhouse. The plants (putative F_1's) were bagged to ensure selfing to produce F_2 seed. Seeds from these plants were planted in 60 cell flats using a commercial greenhouse potting mix (Metro mix) as a growth medium. Two seeds per cell were grown to the one-leaf stage and sprayed with bromoxynil (560 g ai/ha) using a cabinet sprayer. In 1995, 120 seedlings per plant were assessed for resistance or susceptibility to bromoxynil and in 1996, 24 seedlings per plant were assessed. F_2 families which showed an approximate 3:1 ratio of resistance to susceptible were said arise from an outcrossing event (i.e. from a selfed F_1 plant). Putative F_2 families which did not segregate for bromoxynil resistance were assumed to arise from pure breeding bromoxynil resistant plants (i.e. contaminants rather than F_1's). These results were used to confirm if the selected plants were in fact the result of an outcrossing event or if they were pure breeding bromoxynil resistant contaminants.

3.4 STATISTICAL ANALYSES

Data was statistically analyzed using regression procedures which is an appropriate analysis for data with a continuous independent variable (graded levels of a quantitative factor) (Cousens 1988, Morse and Thompson

1981, Petersen 1977). The model that best fit the data was an exponential decay model ($y = ae^{-cx}$). The model was fitted to this data using a derivative-free nonlinear regression procedure (PROC NLIN SAS v.5). R^2 values were calculated as suggested by Kvalseth (1985). Subsequent to regression analysis, to determine whether individual regression lines could be combined, a lack-of-fit F-test was conducted to compare the nonlinear parameter estimates (Ratkowsky 1983, Seefeldt 1995).

To determine an overall outcross mean, an average percent resistant plants per distance/direction/site/year was calculated. This data was statistically analyzed using General Linear Models (GLM) procedures (SAS v.5) for a split-plot design using site-year*direction as the error term to test the significance of site-year and direction. The remainder of the terms were tested using the residual error term.

4. RESULTS AND DISCUSSION

4.1 OUTCROSSING RATE TRIALS CONSIDERED SEPARATELY

Distance from the transgenic pollen source had a major effect on outcrossing rates observed for each site in each year (each environment) in this study (Figures 2 and 3), (Table 2). Rate of outcrossing decreased with increasing distance from the pollen source.

The outcrossing rate to border areas, particularly the inner edge of the border (0 m), varied between years in this study. At 0 m, the 1995 outcrossing rates for each site of the four site-year combinations were approximately double the 1994 outcrossing rates (mean outcrossing rate at 0 m for Carman 94 was 0.56% compared to 1.18% for Carman 95, and for Winnipeg 94 the mean outcrossing rate was 0.39% compared to 0.70% for Winnipeg 95) (Table 3). These values are substantially larger for 1995, although not statistically different according to GLM procedures. Environmental conditions affect pollinator activity in insect pollinated crops (Langridge and Goodman 1982). Since it is unlikely that environmental conditions were identical in the two years of the study, one would expect differences in outcrossing rates between years. This was most evident for Carman where bees were introduced (Figure 2). The 1995 rates were higher than the 1994 rates, suggesting that environmental conditions were more favourable for pollinator activity and therefore, subsequent pollination to occur. However, no data can be presented to verify this hypothesis as no

data was collected on pollinator activity for the 1994 and 1995 trials in either Carman or Winnipeg. Several factors can potentially affect the rate of outcrossing and differences in the rate of pollinator activity is one possibility for differences in outcrossing rates observed for 1994 and 1995.

Outcrossing rates of *B. napus* to border areas may also have been affected by direction in this study, although no consistent direction effect was apparent. In three of four site-year combinations, the west direction had the highest rates of outcrossing and in one site-year combination the east direction had the highest rates of outcrossing (Figures 2 and 3). There is no obvious explanation for the effect of direction. Further studies are necessary to confirm the reproducibility of this direction effect.

Outcrossing rates for all sites and years were low for all directions (less than 1.60%), and declined rapidly to very low levels at 30 m (less than 0.25%) (Figures 2 and 3) (Table 2, Table 3). For Carman 94, Carman 95, Winnipeg 94, and Winnipeg 95 more than four-fifths of the outcross plants were present in the first 10 m of the border area (Table 2, Table 3). Therefore, less than one-fifth of outcross plants detected in this study occurred in the next 20 m of the border area (10 to 30 m distances) (Table 2, Table 3).

4.1.1 OUTCROSSING RATE TRIALS COMBINED OVER SITES AND YEARS

The data for each site and year was combined over directions to obtain an overall perspective of the effectiveness of border areas in

minimizing pollen flow from transgenic *B. napus* trials (Figure 4), (Table 3). For the data, combined over sites, years, and directions, mean outcrossing rates decreased from approximately 0.71% at 0 m to less than 0.07 % at 10 m and further decreased to approximately 0.02% at 30 m (Figure 4), (Table 3). Expressed in frequency of outcross plants per total plants sampled, 1 plant in 140 was the result of an outcross event at 0 m, while 1 plant in 1400 and 1 plant in 5000 were the result of outcross events at 10 m and 30 m, respectively. Figure 4 represents the overall outcrossing rate means for each distance with the range (high and low values) of outcrossing rates shown for each distance. The range bars appear to be greatest at the 0 m distance with less variability at 30 m. However, when the high and low values are considered as a proportion of the mean there is very little variability. The high values are approximately two times greater than the mean value and the low values are approximately one quarter the value of the mean.

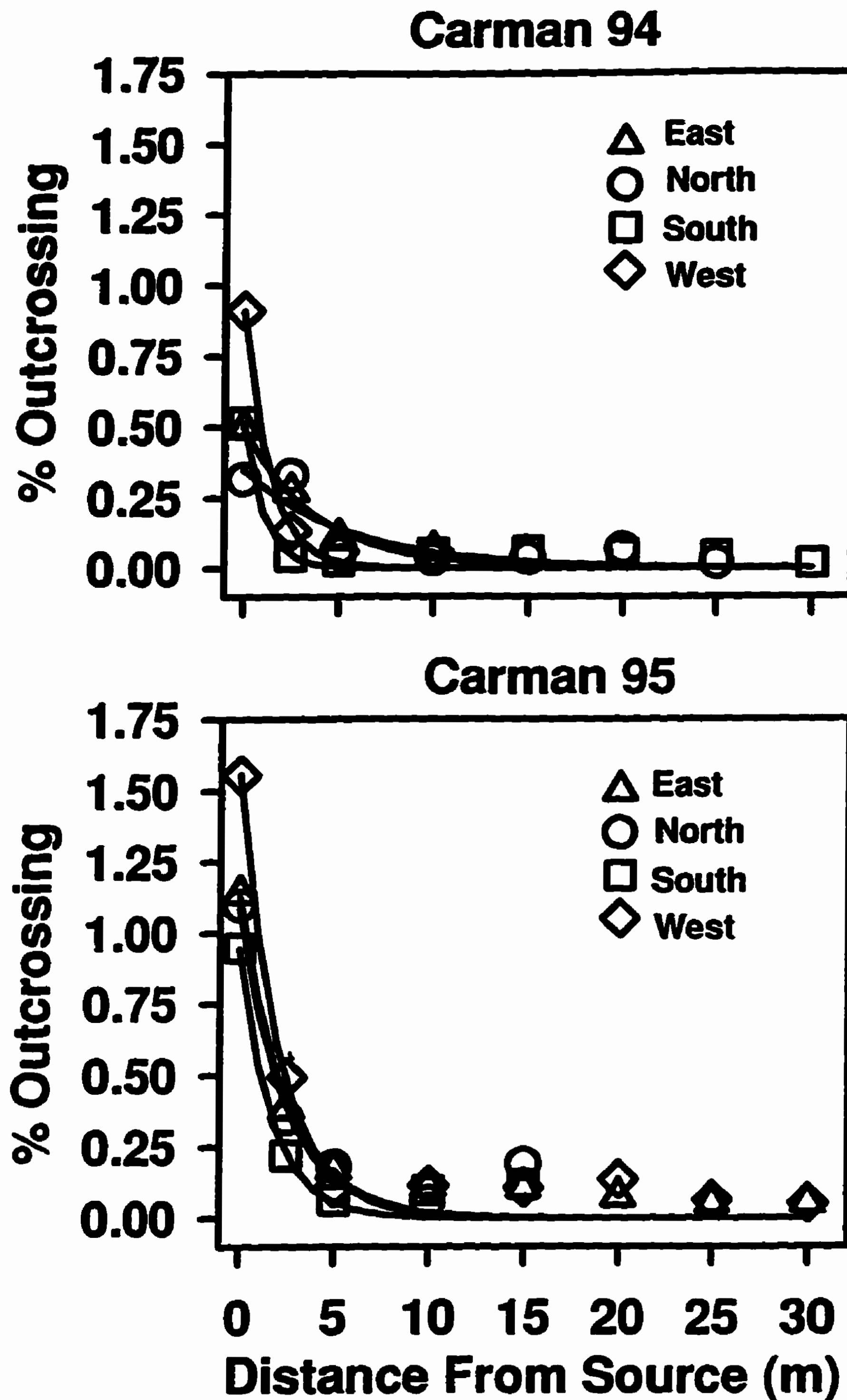

Figure 2. Relationship mean between percent outcrossing and distance from pollen source in transgenic *Brassica napus* outcrossing rate trials using bromoxynil resistance as a marker for outcrossing rate trials grown in Carman in 1994 and 1995.

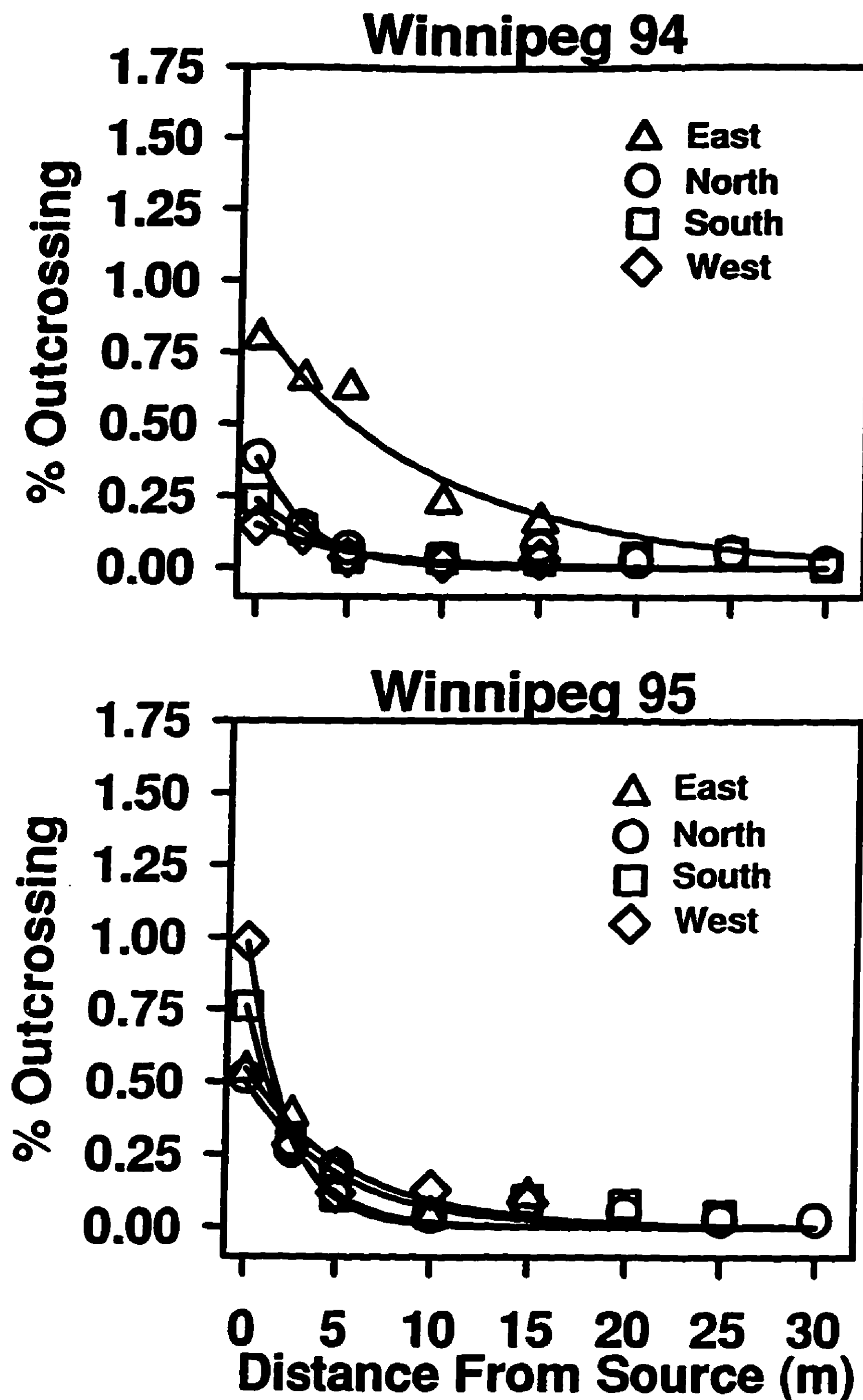

Figure 3. Relationship between mean percent outcrossing and distance from pollen source in transgenic *Brassica napus* outcrossing rate trials using bromoxynil resistance as a marker for outcrossing rate trials grown in Winnipeg in 1994 and 1995.

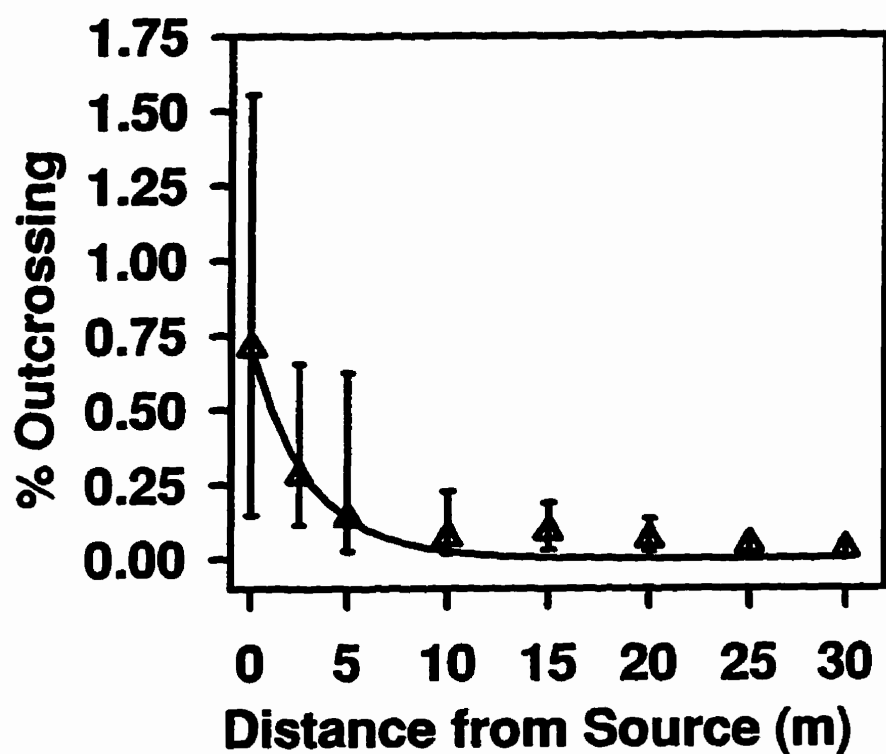

Figure 4. Relationship between percent outcrossing and distance from pollen source in transgenic *Brassica napus* using bromoxynil resistance as a marker. Overall means with high/low range bars for outcrossing rate values for each distance for data combined over sites, years, and directions.

Table 2. Mean outcrossing rates of transgenic *Brassica napus* to border areas in 1994 and 1995 outcrossing rate trials by distance, for Carman and Winnipeg for each direction. (values in parentheses are S.E.)

SITE-YEAR	DISTANCE (m)							
	0	2.5	5.0	10.0	15.0	20.0	25.0	30.0
	% outcrossing							
Carman 94								
East	0.49	0.27	0.11	0.06	....	....	....	....
	(0.22)	(0.15)	(0.03)	(0.01)	....	....	....	....
North	0.32	0.33	0.05	0.03	0.04	0.07	0.02	....
	(0.03)	(0.16)	(0.00)	(0.00)	....	(0.03)	....	....
South	0.52	0.04	0.03	0.05	0.10	0.06	0.05	0.02
	(0.43)	....	(0.01)	(0.01)	(0.06)	(0.02)	....	....
West	0.91	0.13	0.06	0.05	....	....	....	....
	(0.48)	....	(0.00)	(0.03)	....	....	....	....
Carman 95								
East	1.14	0.38	0.17	0.11	0.11	0.07	0.05	0.05
	(0.03)	(0.14)	(0.02)	(0.03)	(0.03)	(0.00)	(0.01)	(0.04)
North	1.09	0.35	0.18	0.05	0.18	....	....	....
	(0.03)	(0.13)	(0.04)	(0.03)	....	....	....	....
South	0.95	0.22	0.06	0.10	0.12	....	....	....
	(0.16)	(0.04)	(0.02)	(0.02)	(0.02)	....	....	....
West	1.56	0.49	0.11	0.11	0.11	0.13	0.06	0.05
	(0.10)	(0.07)	(0.02)	(0.06)	(0.04)	(0.00)	(0.02)	(0.00)
Winnipeg 94								
East	0.80	0.66	0.62	0.25	0.24	....	....	....
	(0.18)	(0.42)	(0.10)	(0.07)	(0.17)	....	....	....
North	0.39	0.14	0.07	0.03	0.06	0.02	0.06	0.02
	(0.16)	(0.08)	(0.03)	(0.01)	(0.03)	(0.01)	(0.03)	(0.01)
South	0.24	0.13	0.04	0.03	0.02	0.04	0.05	0.01
	(0.02)	(0.00)	(0.02)	(0.02)	(0.01)	(0.01)	(0.02)	(0.00)
West	0.15	0.11	0.04	0.02	0.02	....	....	....
	(0.01)	(0.08)	(0.01)	(0.01)	(0.01)	....	....	....
Winnipeg 95								
East	0.54	0.38	0.21	0.04	0.11	....	....	....
	(0.03)	(0.14)	(0.06)	(0.01)	(0.03)	....	....	....
North	0.52	0.26	0.21	0.04	0.08	0.05	0.03	0.03
	(0.19)	(0.03)	(0.12)	(0.00)	(0.03)	(0.02)	(0.00)	(0.01)
South	0.76	0.29	0.11	0.05	0.09	0.08	0.04	....
	(0.19)	(0.15)	(0.01)	....	(0.03)	(0.02)	(0.03)	....
West	0.99	0.28	0.12	0.13	0.09	....	....	....
	(0.03)	(0.17)	(0.04)	(0.00)	(0.02)	....	....	....

Table 3. Mean outcrossing rates of transgenic *Brassica napus* to border areas in 1994 and 1995 outcrossing rate trials by distance, for Carman and Winnipeg combined over directions, for outcrossing rate trials combined over sites and directions and for outcrossing rate trials for combined over years, sites, and directions. (values in parentheses are S.E.)

	DISTANCE (m)							
SITE-YEAR	0	2.5	5.0	10.0	15.0	20.0	25.0	30.0
	% outcrossing							
Carman 94	0.56	0.23	0.06	0.05	0.08	0.06	0.03	0.02
	(0.15)	(0.07)	(0.01)	(0.01)	(0.04)	(0.01)	(0.01)	....
Carman 95	1.18	0.36	0.13	0.09	0.12	0.10	0.06	0.05
	(0.09)	(0.05)	(0.02)	(0.02)	(0.02)	(0.02)	(0.01)	(0.00)
Winnipeg 94	0.39	0.26	0.19	0.08	0.09	0.03	0.06	0.01
	(0.10)	(0.12)	(0.10)	(0.04)	(0.05)	(0.01)	(0.01)	(0.00)
Winnipeg 95	0.70	0.30	0.16	0.07	0.09	0.06	0.03	0.03
	(0.09)	(0.05)	(0.03)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)
1994	0.48	0.25	0.13	0.06	0.08	0.05	0.05	0.01
	(0.10)	(0.07)	(0.05)	(0.02)	(0.03)	(0.01)	(0.01)	(0.00)
1995	0.94	0.33	0.14	0.08	0.11	0.08	0.05	0.04
	(0.09)	(0.04)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Combined	0.71	0.29	0.14	0.07	0.10	0.07	0.05	0.03
	(0.08)	(0.04)	(0.03)	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)

Table 4. Screening results - seedlings screened and resistant individuals for Carman and Winnipeg outcrossing rate trials by distance combined over years and directions

	Carman		Winnipeg	
Distance (m)	Resistant (no.)	Screened (no.)	Resistant (no.)	Screened (no.)
0	2 530	300 000	1 597	300 000
2.5	774	265 000	847	300 000
5	269	291 000	519	282 000
10	243	367 000	264	369 000
15	229	293 000	322	414 000
20	117	140 000	67	140 000
25	61	152 000	63	140 000
30	46	114 000	39	286 000
Total	4 269	1 922 000	3 718	2 231 000

Grand Total Resistant = 7987, Screened = 4 153 000.

Comparisons of the effectiveness of border areas observed in this study versus the effectiveness of isolation distances reported in the literature are possible. However, it is difficult to compare these two methods of pollen control, as insect pollinator activity may affect the observed outcrossing rates for borders areas versus isolation zones. The *B. napus* outcrossing rate into the border area at 10 m distance in this study is significantly lower than for 8 to 10 m isolation zones reported by Bilsborrow et al. (1994) (quantity of pollen in the air after 8 to 10 m isolation distance was reduced to 17.9% of pollen in the air present at the pollen source) or Morris et al. (1994) (approximately 1.5% outcrossing). But, outcrossing rates into the border area at 30 m observed in this study are comparable to outcross rates observed by Scheffler et al. (1995) for a 200 m isolation zone. These results suggest that border areas are much more effective in minimizing *B. napus* pollen flow compared to isolation zones.

Outcrossing rates to border areas from a central transgenic plot of *B. napus* in this study are significantly lower than those reported by Rakow and Woods (1987) (12.5 to 32.8%), Lewis and Woods (1991) (15 to 95%), and Becker et al (1992) (12 to 47%) for interplant within plot outcrossing rates in *B. napus*. Interplant outcrossing within plots can occur through physical contact of the plants, something which could not occur in this study because the transgenic plots and border area were separated by a

rotovated strip. The outcrossing rates observed in this study were also much lower that those observed by Huhn and Rakow (1979 as cited in Rakow and Woods 1987) (5 to 15%) for winter *B. napus* between plot outcrossing rates in Germany. Their results may have been caused by higher levels of insect pollinator activity or the difficulty in measuring large populations of plants for the marker being used in the Huhn and Rakow (1979 as cited in Rakow and Woods 1987) study. For example, in studies in which erucic acid was used as a marker, a form of chromatography was necessary to determine the erucic acid level in the seed. Lewis and Woods (1991) used gas chromatography and paper chromatography to test fewer than 6000 plants for elevated levels of erucic acid. In contrast, in this study over 4 million seedlings were screened for the presence of the bromoxynil resistance trait (Table 4). Larger numbers of plants screened in an outcrossing trial provide a more accurate determination of outcrossing rate. In this study, approximately 8000 plants were the result of outcrossing events in the 4 million seedlings screened to determine rates of outcrossing (Table 4).

The average outcrossing rates to border areas observed in this study are substantially lower (0.71% at 0 m declining to 0.14% at 5 m) than average outcrossing rates to border areas reported by Morris et al. (1994) (2.75% at 0 m declining to 0.65% at 5 m). Again, insect pollinator activity may have been higher in the Morris et al. (1994) trials. Similarly, the

outcrossing rates found by Timmons et al. (1996) in England were significantly greater at 0 m (6.3%) than those found in this study (0.71%). A high rate of outcrossing was reported at 100 m from the pollen source (0.5%) by Timmons et al. (1996). This rate is similar to the levels reported in this study for distances of 0 m from the pollen source indicating much higher rates of outcrossing in the study from England.

The average outcrossing rates to border areas at 30 m observed in this study are also lower (0.02% at 30 m) than those reported by Wilkinson et al. (1995) (0.03 to 0.04% at 32 m) for *B. napus* field trials in Scotland. However, outcrossing rates observed by Dale et al. (1993b) and Scheffler et al. (1993) (0.0041% at 24 m and 0.001% at 36 m) are lower than outcross rates observed in this study (0.02 at 30 m). This also suggests that location of the trial and environmental conditions especially as they affect insect pollinators may affect the amount of outcrossing which can occur from *B. napus* crops to border areas. Conditions favouring outcrossing are high numbers of indigenous pollinators and warm dry conditions as opposed to low numbers of pollinators and cool, wet conditions.

4.1.2 POLLINATORS

The introduction of pollinators (leafcutter bees) at the Carman site did not appear to influence the outcrossing rates in 1994, but may be the reason that the Carman site had generally higher (not significantly in many cases) outcrossing rates in all four directions in 1995 (Figure 2).

4.2 CONFIRMATION TRIAL

In 1995, 28 putative F_1 plants (plants surviving two applications of bromoxynil herbicide in the field) were transplanted into pots which were placed in a growth room, selfed and their progeny (the F_2 families) were screened for segregation with regards to the bromoxynil resistance trait. A segregating family would be the result of an outcrossing event (i.e. progeny of a true F_1 plant) and a non-segregating resistant family would be the progeny of a pure breeding (homozygous) contaminant plant. Results indicated a very high level of contamination in the 28 plants that were screened. Of the 28 transplants, 7 pure breeding individuals were from the 1994-Carman-South-30-Rep 1 plots. These plots had an unusually high percentage of resistant individuals in the field; therefore, a relatively large sample of this population was transplanted to determine the reason for this anomaly. Because there was such a high rate of contamination in these plots, the 1994-Carman-South-30-Rep 1 data was deleted from the data set prior to analysis. Of the remaining 21 plants not from 1994-Carman-South-30-Rep 1, 4 were pure breeding contaminants.

In 1996, 259 putative F_1 plants (plants surviving two applications of bromoxynil herbicide in the field) were transplanted, selfed and screened for segregation of the bromoxynil resistance trait. In 1996, only 14 of the 259 individuals were pure breeding contaminants.

In total, combining both 1995 and 1996 results, 18 of the 280 plants

were pure breeding contaminants. This means that over both years 6.4% of the individuals judged to be resistant in the field (the result of an outcrossing event) were in fact pure breeding contaminants.

The overall data has not been adjusted to account for this contamination rate due to the fact that the percent resistant individuals is low for all sites, years and directions, and the 6.4% contamination rate would have a negligible effect on calculating the true percent outcrossing for any plot. For example, in a plot with an average seedling stand of 6000 plants per plot and 0.50% resistant individuals surviving after bromoxynil application, there would be 30 surviving plants. Based on the 6.4% contamination rate calculated above, approximately 2 of the 30 surviving plants would be pure breeding contaminants and not the result of an outcrossing event. Therefore, the actual outcrossing percent would be 28/6000 or 0.467% (not 0.50%).

Correcting for pure breeding contaminants has a greater impact when the highest rate of percent resistant individuals in this study is considered. In a plot with an average seedling stand of 6000 plants per plot and 1.60% resistant individuals surviving after bromoxynil applications there would be 96 surviving plants. Using the 6.4% contamination rate, 6 individuals would be pure breeding contaminants. In this case, the actual percent outcrossing then is 90/6000 or 1.50% (not 1.60%). There is an apparent greater effect of the correction factor in this example, however the

effect is still relatively minor. In most instances in this study (ie. combinations of year-site-direction-distance), the percent resistant individuals was less than 0.50% and the correction factor, therefore, has a negligible effect in calculating true outcrossing rate.

A potential source for contamination in this study was seed contamination. Seed contamination was possible at numerous times during the study. The pieces of equipment such as seeders and threshers that were used for this study were shared with the bromoxynil resistant cultivar development field program. There was the potential for seed to remain in the seeder or thresher from the bromoxynil resistant cultivar development program and become combined with the seed from this study.

Confirmation of heterozygosity of plants (transplanted survivors, selfed, and screened for F_2 segregation) from the border areas at distances of 25 m and greater confirm that pollen movement by insect pollinators can occur beyond 10 m border areas. For Carman, there were 38 confirmed outcross plants from the 25 m distance and 35 confirmed outcross plants at 30 m. For Winnipeg, there were 39 confirmed outcross individuals at 25 m, 8 confirmed at 30 m and 6 confirmed at 35 m.

4.3 STATISTICAL ANALYSES

The exponential decay model for outcross rate as a function of distance fit the data very well, as indicated by the high R^2 values for all directions at both sites in both years (Table 5). The exponential decay

model provided a much better fit to the data than the Weibull model (data not shown), another commonly used probability distribution for this type of data.

Lack-of-fit F-test indicated that while certain parameter estimates or lines could be combined, others could not, therefore the data is presented separately by site, year and direction (Figures 2 and 3).

Subsequent General Linear Model (GLM) analysis indicated that only the main factor distance, was statistically significant (Table 6). The distance from source represented approximately 50% of the treatment sums of squares for main factors and their interactions and was, therefore, the primary factor affecting outcrossing. Although GLM was the only apparent choice of a statistical procedure to distinguish differences between year, site and direction, and other researchers have followed this approach (Seefeldt 1995), it should be remembered that the regression model that best fit the data was nonlinear and GLM results should be interpreted with caution.

Table 5. Exponential decay model $y=ae^{-cx}$ parameter estimates for percent outcrossing of transgenic *Brassica napus* to border areas in 1994 and 1995 by site, year and direction (values in parentheses are S.E.) In the model y=percent outcrossing, x=distance from pollen source (m), c=nonlinear coefficient determining slope of curve, a=intercept at 0 m distance and e=base of natural logarithm

Site-Year	Direction	a (intercept)	c (slope)	R^2
Carman 94	East	0.50 (0.03)	-0.26 (0.03)	0.99
	North	0.35 (0.07)	-0.18 (0.07)	0.78
	South	0.52 (0.05)	-0.96 (0.39)	0.93
	West	0.91 (0.05)	-0.73 (0.13)	0.99
Winnipeg 94	East	0.84 (0.07)	-0.10 (0.02)	0.93
	North	0.38 (0.04)	-0.35 (0.08)	0.91
	South	0.24 (0.03)	-0.26 (0.08)	0.85
	West	0.15 (0.02)	-0.19 (0.05)	0.87
Carman 95	East	1.13 (0.07)	-0.40 (0.06)	0.97
	North	1.09 (0.11)	-0.41 (0.10)	0.94
	South	0.94 (0.09)	-0.57 (0.14)	0.96
	West	1.56 (0.09)	-0.47 (0.07)	0.97
Winnipeg 95	East	0.55 (0.05)	-0.18 (0.04)	0.95
	North	0.50 (0.04)	-0.20 (0.03)	0.95
	South	0.76 (0.06)	-0.37 (0.06)	0.96
	West	0.98 (0.09)	-0.46 (0.10)	0.96
Combined		0.70 (0.05)	-0.33 (0.06)	0.95

Table 6. General linear model (GLM) analysis for outcrossing rate trials

Factor	Df	Sum of Squares	F-statistic
Site-year	3	1.1302	2.38 NS
Direction	3	0.6544	1.38 NS
Distance	1	4.4800	81.28 ***
Site-year*Direction (error a)	9	1.4240	
Site-year*Distance	3	0.5268	3.19 *
Direction*Distance	3	0.4252	2.57 NS
Site-year*Direction* Distance	9	1.1981	2.42 *
Error b	162	8.9295	

NS = non-significant, * ** *** significant at p=0.05, 0.01 and 0.001 respectively

5. CONCLUSIONS

The purpose of this study was to determine the effectiveness of border areas in limiting pollen flow from transgenic *B. napus* field trial plots in western Canada. It was determined that outcrossing to border areas was relatively low for all years, sites and directions observed in this study. Outcrossing rates declined rapidly from 0 to 5 m and continued to decline more slowly at distances greater than 5 m. Overall outcross rates for years, sites and directions combined averaged at 0.70% at 0 m and declined to less than 0.02% at 30 m.

The introduction of pollinators to half of the outcrossing trials (leafcutter bees) did not appear to significantly influence outcrossing rates.

No consistent direction effect on outcrossing rates was observed in the study.

The majority (over 80%) of outcrossing observed in the study occurred in the first 10 m of the non-transgenic *B. napus* border areas around transgenic *B. napus* plots. Therefore, the border areas were effective in containing the majority of transgenic *B. napus* pollen. However, complete containment of all *B. napus* pollen from the transgenic field trials is not possible due to the fact that *B. napus* can be cross-pollinated by insects. Therefore, the potential for rare long distance pollen transfer by pollinators exists.

6. IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

This study indicates that pollen movement in *B. napus* follows a basic trend in which outcrossing is greatest near the pollen source and decreases with increased distance from the pollen source. The exponential decay model fitted the data well indicating that this trend should be repeatable in future field trial experiments and for field situations. Since distance is the overriding factor affecting outcrossing, it should be possible to estimate or predict potential outcrossing in future trials. Site and year did not have significant effect on outcrossing therefore, it should be possible to use the outcrossing model from this study to predict potential outcrossing for transgenic *B. napus* being grown in Western Canada.

This study also suggests that pollinators of *B. napus* will move pollen within the given area of the pollen source and surrounding sexually compatible plants (i.e. *B. napus* surrounding the pollen source). Appendix Table 3 indicates that outcrossing occurred and was confirmed to have occurred up to 60 m from the pollen source. Therefore, if *B. napus* crops are near other *B. napus* crops, there is the potential for pollen to move great distances.

This experiment creates the potential for further experiments to be developed to expand the information which was gathered in this study. One such study should include the determination of outcrossing rates to non-transgenic plots within the transgenic field trial. These rates could be

compared to the rates of outcrossing occurring in the border areas. The non-transgenic plots would be surrounded on all sides by transgenic plots. The objective of the study would be to determine if being completely surrounded by the transgenic pollen source increases outcrossing. It would also be possible to determine if a rotovated strip between the trial and border affects outcrossing. In another study currently being conducted at the University of Manitoba using bromoxynil resistance as a marker, average outcrossing to internal non-transgenic plots within the transgenic trial is approximately 5.0% (Janice Cuthbert, pers. comm.). This is significantly greater than the rates measured at 0 m in the border in this study, suggesting that being completely surrounded by the transgenic pollen source increases the rate of outcrossing and that a rotovated strip may decrease the amount of outcrossing occurring. Pollinator activity could also be monitored in an attempt to determine if the rotovated strip around the trial has any affect on the movement of pollinators.

Novel herbicide resistant transgenic crops are currently being grown in large agricultural scale fields. This creates another opportunity for studying outcrossing in transgenic *B. napus*. Will the outcrossing models observed for transgenic field trials be useful in determining outcrossing rates from fields of transgenic *B. napus* to neighbouring fields of non-transgenic *B. napus*? Studies should be conducted to determine levels of outcrossing from transgenic *B. napus* field to adjacent non-transgenic *B.*

napus fields. In such a study the fields will border each other and the edge of this bordering side should be considered the 0 m distance into the adjacent non-transgenic field. Outcrossing should be measured at numerous distances from the pollen source (transgenic field). At least two samples from each distance should be collected. In order to compare rates with the rates observed in field trials, samples should be taken at 0, 2.5, 5, and 10 m. Additional samples should be taken every 10 to 20 m to the edge of the field furthest from the transgenic pollen source. Screening the samples to determine rates of outcrossing will involve large numbers of plots and seedlings being screened. The greater distances should have low rates of outcrossing and therefore, greater numbers of seedlings may have to be screened in order to discover the rare outcross individual. If the outcrossing is approximately 1.0% at the 0 m distance, 5000 seedlings screened will result in approximately 50 outcrossing individuals being detected. At the greater distances, outcrossing rates may be around 0.01%. In order to detect 5 outcrossing individuals at this rate of outcrossing over 50 000 seedlings would have to be screened.

Field scale trials offer the opportunity to introduce a random pollinator such as honeybees to determine if there is an affect of the introduction of these pollinators. This trial would require at least two locations, one with introduced pollinators and one without the introduced pollinators. Outcrossing rates would be determined as stated previously.

Future trials should focus on determining pollen movement in *B. napus*. As the numbers of transgenic varieties increase, it will be necessary to try to prevent multiple gene introgression from occurring in a single plant and thus creating a plant for which there are few known herbicidal controls. This would pose numerous problems for the agriculture industry and should be avoided at all costs.

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APPENDIX

An additional transgenic *B. napus* outcrossing rate field trial was established in 1995 at the I.C.M.S. Research farm at Portage la Prairie, Manitoba (Appendix Table 1). Outcrossing rates were extremely low for all directions and distances. Neither the exponential decay model nor the Weibull model fitted the data. The outcrossing rates for all directions by distance are presented in Appendix Table 2. It is unknown why outcrossing rates were so low. Low outcrossing rates may have resulted from the widespread use of insecticides to control insect problem in potatoes in the Portage la Prairie area thereby reducing the number of insect pollinators. Also, the borders were severely lodged and this may have adversely affected pollinator activity and outcrossing rates. Confirmation of heterozygosity of plants (transplanted survivors, selfed and screened for F_2 segregation) at distances greater than 25 m confirm that insects moved the transgenic *B. napus* pollen (Appendix Table 3).

Appendix Table 1. Plots seeded, seeds planted and seedlings screened (combined over directions)

Distance (m)	Portage la Prairie Plots (no.)	Seeds (no.)	Seedlings (no.)
0	24	199 000	140 000
2.5	24	199 000	140 000
5	24	199 000	140 000
10	18	149 000	105 000
15	18	149 000	105 000
20	12	100 000	70 000
25	6	50 000	35 000
30	6	50 000	35 000
38	6	50 000	35 000
40	6	50 000	35 000
45	6	50 000	35 000
50	6	50 000	35 000
55	6	50 000	35 000
60	6	50 000	35 000
65	6	50 000	35 000
70	6	50 000	35 000
75	6	50 000	35 000
Total	180	1 495 000	1 050 000

Appendix Table 2. Outcrossing rates of transgenic *B. napus* to border areas in 1995 for Portage la Prairie by direction and for directions combined (values in parentheses are S.E.)

	DISTANCE (m)					
Direction	0	2.5	5.0	10.0	15.0	20.0
	% outcrossing					
East	0.17	0.07	0.06	0.02	0.03	0.01
North	0.11	0.03	0.02	0.01	0.01	....
South	0.25	0.17	0.05	0.07	0.05	0.03
West	0.13	0.05	0.03	....	....	....
Combined	0.17	0.08	0.04	0.03	0.03	0.02

(cont)

	DISTANCE (m)					
Direction	25.0	30.0	38.0	45.0	50.0	55.0
	% outcrossing					
East	0.01	0.01	0.00	....	....	....
North	....	....	....	....	....	....
South	....	....	....	....	....	....
West	....	....	....	0.01	0.01	0.00
Combined	....	....	....	....	....	....

(cont)

	DISTANCE (m)			
Direction	60.0	65.0	70.0	75.0
	% outcrossing			
East	....	....	....	....
West	0.00	0.00♦	0.00	0.00
South	....	....	....	....
North	....	....	....	....
Combined	....	....	....	....

♦ Percent outcrossing = 0.006

Appendix Table 3. Confirmed outcross individuals (by segregation of F_2 families) 25 meters and beyond for Portage la Prairie for combined directions

Distance (m)	Confirmed Outcross (no.)	Seedlings Screened (no.)
Portage la Prairie		
25	5	35 000
30	4	35 000
45	6	35 000
50	5	35 000
65	2	35 000