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Transgenic Bromoxynil Resistant Oilseed Rape: Biological Cost and Outcrossing Studies

By

Janice Louise Cuthbert

A Thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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TRANSGENIC BROMOXYNIL RESISTANT OILSEED RAPE: BIOLOGICAL COST AND OUTCROSSING STUDIES

BY

JANICE LOUISE CUTHBERT

A Thesis/Practicum 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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Transgenic bromoxynil resistant oilseed rape: biological cost and outcrossing studies

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

Bromoxynil herbicide resistance in oilseed rape (*Brassica napus* L.) is conferred by transgenic insertion of a single gene from a soil bacterium that confers the ability to metabolize hydroxybenzonitrile herbicides such as bromoxynil. The level of resistance of bromoxynil herbicide is high but it is unknown whether there is a biological cost associated with this resistance gene or the derived herbicide resistance in oilseed rape. The performance of two isogenic transgenic bromoxynil resistant populations, Westar 235 and Westar 237, and one transgenic bromoxynil resistant line, 235 245 derived from Westar were evaluated. All were either sprayed with bromoxynil at 280 g a.i./ha or left unsprayed and were compared to the nontransgenic population, Westar, in Winnipeg, Carman, and Portage la Prairie from 1994 through to 1997. There were no consistent differences in the performance of the sprayed or unsprayed transgenic populations or the transgenic line in comparison to Westar for lodging, plant height, days to flower, days to maturity, yield, oil content, protein content, or sum of oil and protein content measured in this study. There is very little evidence of any biological cost associated with bromoxynil resistance in oilseed rape, even when the plants are sprayed with bromoxynil. Indeed, there were

significant biological benefits associated with bromoxynil resistance, especially when the plants were sprayed with bromoxynil herbicide.

The bromoxynil resistance gene can be used as a very accurate and definitive marker to measure unidirectional pollen mediated gene flow (outcrossing) in oilseed rape because it is inherited as a single, nuclear, dominant gene. Unidirectional outcrossing rates were assessed between neighbouring plots and rows of oilseed rape in a typical plant breeding field situation, specifically, a plot to plot trial, a row to row trial (40, 80, and 120 cm row spacings), and a plot to plant trial. Bromoxynil susceptible (pollen recipient) plants, plots and/or rows were planted and surrounded by bromoxynil resistant (pollen donor) plots or rows. Outcrossing rate field trials were conducted at Winnipeg, Carman, and Portage la Prairie in 1996 and 1997. Only the seed produced on the susceptible plants, plots and rows was harvested. The harvested seed was planted the following spring in the field and seedlings were sprayed with 560 g a.i./ha of bromoxynil to identify resistant individuals. A total of 419,527 seedlings were screened with 23,816 resistant individuals identified, each resistant individual being the result of an outcrossing event. The maximum rate of outcrossing was observed in the plot to plant trials and ranged from 6 to 81% on an individual plant basis with an overall mean of 21.0% (±1.73). The mean rate of outcrossing in the other planting arrangements varied from 9.5% (±0.62) for the 40 cm row spacing to 3.9% (±0.25) for the 120 cm row spacing.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 INTRODUCTION

Canola quality oilseed rape (*Brassica napus* L.) is an important crop grown worldwide as a source of edible vegetable oil. The acreage of canola has increased steadily in western Canada since the first low erucic, low glucosinolate cultivar, Tower, was released from the University of Manitoba in 1974 (Eskin *et al.* 1996). Currently, annual production in western Canada has been estimated at seven million tonnes with a value over two billion dollars (Statistics Canada 1997).

Canola has become an important crop because it is amenable to various crop development techniques including traditional and genetic engineering. Traditional breeding methods are used to select and incorporate superior traits into existing cultivars or develop new cultivars (Poehlman and Sleper 1995). The desirable traits can only be transferred between sexually compatible species. Genetic engineering permits researchers to transfer a desirable trait, encoded by one or more genes, from one organism to another using insertion technology (Poehlman and Sleper 1995). This technology eliminates the need for the organisms to be sexually compatible. The result of the gene transfer is a transgenic plant with a novel trait such as herbicide resistance.

Currently, three types of genetically modified herbicide resistant canola are available for producers to grow commercially. In 1997, herbicide resistant canola

accounted for 50% of the canola acreage in western Canada and estimates for 1998 indicated the acreage would increase to over 65% (NRC 1997).

Herbicide resistant crops provide better weed control by allowing producers to reduce the use of herbicides and switch to more environmentally safe broad-spectrum herbicides (Stalker *et al.* 1996). However, researchers are concerned the incorporation of novel herbicide resistance in oilseed rape could affect the phenotype and/or agronomic performance of the transgenic crop by disrupting some essential functions or by diverting energy and limited resources to the novel herbicide resistance (Hails *et al.* 1997; Bergelson *et al.* 1996; Mallory-Smith and Eberlein 1996; Arnoldo *et al.* 1992). Plant injury can also occur if the herbicide resistance is incomplete. These negative effects on the crop phenotype or performance are referred to as biological cost.

A significant biological cost had been documented for triazine herbicide resistance in canola, the first herbicide resistant crop available to farmers. Triazine resistant varieties were developed by transferring the naturally occurring resistance from *B. rapa* to *B. napus* using traditional breeding methods. The presence of the triazine resistant cytoplasm in triazine resistant varieties reduced yield by 20 to 30% and delayed flowering by two days when compared to triazine susceptible canola isogenic populations (Beversdorf and Kott 1987; Holt *et al.* 1993; Beversdorf *et al.* 1988). The production of triazine resistant cultivars was

only cost effective in areas where the pressure from related weeds like wild mustard was high (Gronwald 1994).

A new type of herbicide resistant canola is under development at the University of Manitoba. A nitrilase gene cloned from a soil bacterium and incorporated in *B. napus* via an *Agrobacterium tumefaciens* mediated transformation system (Stalker *et al.* 1988; Stalker and McBride 1987) confers bromoxynil herbicide resistant in canola. Chemically grouped as a hydroxybenzonitrile, bromoxynil is used to control dicotyledonous weeds in monocotyledonous/cereal crops (Freyssinet *et al.* 1996). Monocots naturally possess a nitrilase gene capable of metabolizing bromoxynil herbicides into non-phytotoxic benzoic acid forms (Stalker *et al.* 1988). It is possible canola could suffer a biological cost as a result of inserting the nitrilase gene or as a result of conferred bromoxynil resistance. This study will attempt to determine if there is a biological cost related to transgenic bromoxynil resistance.

Oilseed rape is a primarily self-pollinating crop that possesses sticky, entomophilous pollen well suited to the transfer by insects (Eisikowitch 1981). Given the relatively low population of insect pollinators in western Canada, it is presumed that the outcrossing rate of oilseed rape in field nurseries and yield trials is low enough to be safely ignored. The outcrossing studies completed to date have used a number of phenotypic traits such as stem color and petal color or oil quality characteristics such as erucic acid content to estimate the

outcrossing rate of oilseed rape. These studies observed oilseed rape exhibits an average outcrossing rate of 20 to 40% (Becker *et al.* 1992; Rakow and Woods 1987; Gowers 1981; Olsson 1960) which indicates oilseed rape frequently outcrosses within its species. Outcrossing rates in this range would lead to rapid contamination of breeding lines and to unexpected genetic segregation in a field based oilseed rape plant breeding program.

The results of the previous outcrossing studies are not definitive or accurate since a number of the markers are influenced by the environment or by other genes. The use of a transgenic herbicide resistance marker such as bromoxynil resistance is easy and more accurate, since the resistance is inherited as a single, nuclear dominant gene (Stalker *et al.* 1996). Bromoxynil herbicide resistance provides a unique tool to accurately assess the outcrossing rates of summer oilseed rape in typical plant breeding field layouts in western Canada.

2.0 LITERATURE REVIEW

2.1 OILSEED RAPE

The Brassicaceae family contains several important crop species including *Brassica napus* L. (oilseed rape). Grown as a source of edible oil, oilseed rape has become the most valuable crop to producers. In western Canada in 1997, 7 million tonnes of oilseed rape were produced on 4.7 million hectares with a value of 2.7 billion dollars (Statistics Canada 1997).

2.1.1 Breeding Methods

The breeding system of a crop, self- or cross-pollinating, determines the methods a plant breeder uses to select and incorporate desirable traits into existing cultivars or develop new cultivars. Oilseed rape is a predominately self-pollinating crop (Olsson 1960) which exhibits an average outcrossing rate between 20 and 40% in Europe (Becker *et al.* 1992). Despite the outcrossing rates quoted in the literature, canola breeders in Canada and elsewhere use breeding methods appropriate for a self-pollinating crop.

These methods include: pedigree, bulk population, single seed descent, doubled-haploid, backcross, and recurrent selection (Thompson and Hughes 1986; Buzza 1995; Poehlman and Sleper 1995). Pedigree, bulk population, and single seed descent are common selection procedures used to identify desirable genotypes from segregating populations following hybridization, while recurrent selection is

a population improvement procedure designed to increase the frequency of desired genes by repeated cycles of selection (Poehlman and Sleper 1995). Backcross breeding allows the transfer of a simply inherited trait from a poorly adapted donor line into a well-adapted cultivar (Buzza 1995).

In all breeding methods, two parental lines with desirable traits are identified and crossed by hand. At this point, the selection and seed handling practices between the F_2 and F_8 generations differ. Each generation of self-pollination decreases the degree of heterozygosity and after five to eight generations, the progeny will be uniform in appearance and performance. The time required to achieve homozygosity can be reduced by the use of doubled-haploids (Poehlman and Sleper 1995; Knowles 1989). This procedure generates haploid plants from the pollen grains produced on the anthers of F_1 plants. The chromosomes of the haploid plants are subsequently doubled with colchicine to produce diploid plants. The doubled-haploids are completely homozygous at all loci, which can eliminate up to 6 generations from a conventional breeding program (Poehlman and Sleper 1995; Knowles 1989).

Pedigree selection is the most widely used method for developing cultivars in self-pollinated crops. Evaluation of individual plants begins in the F_2 generation. Superior plants with the desired traits from both parents are selected and harvested individually. Being a self-pollinating crop, oilseed rape is presumed to have a high level of homozygosity, so single plant selection can be practiced and

progenies will breed true or almost true to type (Knowles 1989; Halloran and Luckett 1994). The F_3 to F_5 generations can be studied in the greenhouse or in progeny rows in the field. Selections are made between and within progeny rows. During the F_6 or F_7 generation, the superior experimental lines are evaluated in a preliminary yield trial and compared to appropriate with check cultivars. The best lines are advanced to replicated yield trials at two or more locations and are again compared to appropriate check cultivars.

Greenhouse selections are usually bagged to ensure self-pollination while field selections are not. It is assumed the rate of outcrossing and genetic exchange in the field for important agronomic and quality traits is minimal and the next generation will breed almost true to type. Like genotypes are grouped together in the field to reduce genetic drift for important agronomic and quality characteristics.

2.1.2 Reproductive Biology

2.1.2.1 Flower Morphology

Oilseed rape has a perfect, radially symmetrical flower with four sepals, four yellow petals, an inner whorl of four longer stamens and an outer whorl of two shorter stamens, and four nectaries (Eskin *et al.* 1996; Williams 1985; Eisikowitch 1981). Two of the nectaries are found between the longer and shorter stamens and are referred to as the inner nectaries. The other two or outer nectaries are located between the shorter stamens and the petals. The ovary has

two united carpels (Williams 1985). The nectaries secrete an abundance of nectar with various sugar concentrations. The inner nectaries begin producing nectar before the flower opens and will usually produce more than the outer nectaries (Williams 1985). The nectar is very attractive to insects, particularly bees. Hammer (1952 as cited by Free 1993) reported bees would visit fields 3.5 to 4 km from their hives in order to collect oilseed rape nectar.

Oilseed rape flowers usually open early in the morning (4:00 to 6:00 a.m.) and most flowers are open by 9:00 a.m. (Williams 1985). The flowers remain open for 1 to 3 days, closing at night. The flowers begin to close between 5 and 6 p.m. and are nearly closed by midnight. The stigma is receptive to pollen approximately three days before the flowers open and it remains receptive for approximately 6 days (Downey and Robbelen 1989).

Pollen release also begins before the flower opens. When the flowers first open, the stigma is located below the shorter stamens. During flowering, the style extends the stigma to the height of the longer stamens (Williams 1985; Free 1993). The shorter stamens dehisce pollen inwards while the longer stamens dehisce outwards. At the end of anthesis, the anthers of the longer stamens curve towards the stigma (Williams 1985; Free 1993; Eskin *et al.* 1996). Insects are required to transfer the pollen from the anthers of the shorter stamens while the action of the wind likely helps to transfer the pollen from the longer stamens (Free 1993). The flowering period of this indeterminate crop is 3 to 5 weeks

depending on genotype and environmental conditions (Eskin *et al.* 1996).

2.1.2.2 Pollen Characteristics

Oilseed rape flowers produce a large supply of pollen. Each anther produces pollen on three oval/round furrows approximately 27 µm long (Kirk 1996). Mesquida and Renard (1982) reported each flower can produce about 1 mg of bright yellow, sticky pollen. The pollen grains are usually found in clusters and easily adhere to the body and legs of pollinating insects. Oilseed rape pollen has a high nutritive value for bees, it contains about 4.9% nitrogen (Louveaux 1958 as cited by Mesquida and Renard 1982).

Williams (1984) studied the concentration of air-borne pollen over a crop of flowering Oilseed rape using an automatic volumetric suction trap. A range of 7.5 – 5295 pollen grains/m^3 of air and an average of 646 grains/ m^3 during the 39 day trapping period was observed. The amount of pollen trapped varied depending on the environmental conditions as well as the growth stage of the crop. The amount of airborne pollen above the crop was presumed to be considerable suggesting wind could be important in the pollination of oilseed rape. However, the characteristics of pollen indicate oilseed rape is not a primarily wind pollinated species. Wind pollinated species are characterized by their smooth, dry pollen grains which are readily dispersed unlike the sticky, entomophilous characteristics of oilseed rape pollen grains (Eisikowitch 1981).

To date, little research has been conducted on the viability or longevity of

oilseed rape pollen. Chiang (1974) reported oilseed rape pollen remains completely viable up to 50 days if stored in damp petri plates at 4°C. Based on this research, it is difficult to estimate the length of time oilseed rape pollen would remain viable in the field.

2.1.2.3 Pollination Mechanisms

The transfer of pollen grains from the anther to the stigma of a plant is known as pollination. In order to achieve pollination a sequence of events must occur: flowers must be open, anthers must release pollen, pollen vectors (pollen transfer agents such as gravity, wind, and insects) must be active, stigma must be receptive, and pollen must be capable of germination and fertilization (Frankel and Galun 1977).

2.1.2.3.1 Self-Pollination

Self-pollination refers to the transfer of pollen from the anthers to the stigma of the same plant (Poehlman and Sleper 1995). This transfer occurs by wind or insects. Auto-pollination is a type of self-pollination where pollen is transferred from the anthers to the stigma of the same plant without the aid of pollinating agents (Williams 1978). Jenkinson and Glynne –Jones (1953) and Williams (1978), studied auto-pollination of several oilseed rape cultivars in the relatively still air of the greenhouse. In both studies only a third to half as much seed was set by auto-pollination as by mechanical self-pollination.

2.1.2.3.2 Sib-Pollination

Sib-pollination or sib-mating is the mating of close relatives (Poehlman and Sleper 1995). This method of pollination occurs when pollen grains from one plant are transferred to the stigma of another plant that is genetically related. This transfer usually occurs by wind or insects.

2.1.2.3.3 Cross-Pollination

Cross-pollination, sometimes referred to as outcrossing, is the transfer of pollen grains from the anther of one plant to the stigma of another plant with a different genetic constitution (Poehlman and Sleper 1995). This transfer of pollen usually occurs by wind or insects.

2.1.2.3.4 Outcrossing

Outcrossing, also known as the natural rate of crossing, is defined by Grant (1975) as the percentage of a progeny of a given individual or biotype grown in a mixed planting and exposed to open pollination, that is derived from the pollen of a different genotype.

The amount of self- and cross-pollination varies and depends on the cultivar or line, environment (temperature, humidity, precipitation), velocity and direction of wind at the time of pollination, and the number and activity of pollinating insects (Poehlman and Sleper 1995).

2.1.2.4 Pollination Agents

2.1.2.4.1 Wind

Wind plays an important role in the pollination of oilseed rape (Olsson 1955 as cited by Mesquida and Renard 1982; Williams 1978, Williams 1984; Eisikowitch 1981; Mesquida and Renard 1982; Free 1993). It is believed the wind works in two ways: pollen is transferred by wind movement of plants which favors self-pollination (Williams 1978; Eisikowitch 1981) or wind transports pollen throughout the crop favoring sib- or cross-pollination (Olsson 1955 as cited by Mesquida and Renard 1982; Mesquida and Renard 1982). In two separate studies by Persson (1956 as cited by Free 1993) and Olsson (1955 as cited by Free 1993) it was concluded wind was the main pollinating agent of oilseed rape since a five-fold difference in bee populations did not affect the percent outcrossing of two oilseed rape cultivars.

Olsson (1955 as cited by Mesquida and Renard 1982) reported oilseed rape pollen could be transported by wind as far as 40 m depending on the cultivar studied. Mesquida and Renard (1982) completed a similar study to Olsson using male fertile and male sterile lines in open and caged plots. Pollen collectors were set up at various distances throughout the strips of oilseed rape. Pollination was confirmed in the male sterile plots by measuring the fruit set rates (number of pods divided by the total number of flowers multiplied by 100) and seed production. They recovered pollen grains up to 32 m from the male fertile strips. However, 75% of the pollen was captured within the first 6 m and 7 to 10% was

captured between 24 and 32 m. They also noted the pollen transferred over long distances did not influence fruit set in the cages. When fruit set was compared between the caged plots and the open pollinated plots, only 23 to 29% of the seed production in the first 6 m was attributed to the wind.

Williams (1978) simulated wind pollination by shaking oilseed rape plants daily. The shaken plants set more seeds per pods, more pods per plant and heavier seeds than the auto-pollinated plants. She also studied the effect of hand pollination (self- and cross-) on seed set and production and compared the results to the shaken plants. The hand self- and cross-pollinated plants produced more seeds per pods, more pods per plant and heavier seeds than the shaken plants. Since the shaken plants produced more seeds than the auto-pollinated plants, Williams concluded the movement of the plants by wind improves the self-pollination of oilseed rape cultivars.

Eisikowitch (1981) studied the effect of wind in a number of greenhouse studies. In one study, oilseed rape flowers were subjected to a range of wind velocities (0 to 5 m/sec) of a hair dryer in an attempt to release pollen grains from their anthers. The air velocities in this study were unable to dislodge pollen grains; however, small clouds of pollen were released when the anthers were touched with a brush or needle. Similar pollen clouds were also observed when insect pollination was studied. The bees created a cloud of pollen when they left the flower after nectar collection but only on dry days. Eisikowitch (1981) concluded

the pollen clouds created by insects, released the only pollen that is truly dispersed by wind.

2.1.2.4.2 Insects

Oilseed rape is an attractive crop to insect pollinators. The flowers produce an abundant nectar supply which insects actively collect. As they move from flower to flower and plant to plant to collect more nectar, they inadvertently become dusted with pollen while visiting the nectaries. The insects also touch the stigma, depositing this pollen. Even though the pollination of oilseed rape has been extensively studied the influence of insect pollinators on crop development and yield remains unclear. Insect pollinators are believed to increase seed set (Eisikowitch 1981), decrease plant height (Barbier 1977 as cited by Mesquida and Renard 1981), increase yield (Eisikowitch 1981), produce longer pods (Ewert 1929 as cited by Mesquida and Renard 1981; Eisikowitch 1981) and produce more pods per square foot (Meyerhoff 1954 as cited by Mesquida and Renard 1981; Eisikowitch 1981). In general, researchers agree the length of flowering can be reduced if insects pollinate oilseed rape. Ewert (1929 as cited by Mesquida and Renard 1981) and Eisikowitch (1981) observed rapid, uniform pod development in plots pollinated by insects. The plots also matured quicker. Barbier (1977 as cited by Mesquida and Renard 1981) indicated flowers pollinated by bees had a life span of three days, compared to five days if unpollinated.

Williams *et al.* (1987) completed several replicated experiments to determine the effect of insect pollination on plant development and seed production. Plots caged to exclude insects were compared to plots caged with a honeybee colony and open pollinated plots. The plots with honeybees consistently flowered for a shorter period of time, shed their petals sooner, and had more advanced phenological development than the plots without bees. The presence of honeybees increased the number of pods per plant and seeds per pod. The pods contained 31% more seeds than pods from the plants without bees, however, the increase in seeds per pod was not reflected in the final yield of the plots. Further investigation of seed development demonstrated the bees increased the number of fertilized ovules in the ovary but the proportion that developed into mature seeds was determined by the growing conditions. Williams *et al.* (1987) concluded honeybees were effective pollinators of oilseed rape.

Sihag (1986) assessed the role of natural insect pollinators on seed production of oilseed rape. Quantitative data of different yield parameters were collected and compared between the open and caged plots. A 34% increase in pod length and a 36% increase in the number of seeds per pod was observed in the insect pollinated plots. Seed yield data was not presented but the author indicated the presence of insects increased yield about 1.8 times per unit area.

Free and Nuttall (1968) studied the effect of bee visits on pollination. They set up

a replicated experiment with three treatments. Natural pollinators pollinated one third of the plots, one third were caged to exclude bees, and the remaining one third were caged with tunnels to beehives just outside the cages. The caged plots with bees produced 13% more seed, however, no significant differences were detected between treatments. The mean 1000 seed weight was significantly higher in the caged plots without bees probably because there were fewer seeds per pod to mature.

Mesquida and Renard (1981) studied the effect of honeybees in male fertile and male sterile oilseed rape. They considered the phenological features such as fruit set rate and plant height and yield parameters like number of pods, number of seeds per pod and seed yield. Each plot contained three male fertile and three male sterile strips, three meters long. The caged plots measured 3x3x2 m or 18 m^3. Plots in cages with honeybees (one hive, 2 frames) and plots in cages without honeybees were compared to determine the effect of insect pollination on development and yield of oilseed rape. The influence of the honeybees was observed in the male sterile plants. Plant height decreased 6 cm and the flowering period decreased 2 days when compared to the caged male sterile plots without honeybees. Fruit set rates increased 17%, number of pods per plant increased 63% and number of seeds per pod increased 29% when compared to the plots caged without bees. Seed yield per plot increased 25% in the caged plots with honeybees. For all the parameters measured, the differences between the caged plot with honeybees and the caged plots without

honeybees were significant. Mesquida and Renard (1981) concluded honeybees positively contribute to the development and yield of oilseed rape.

The research on insect pollination of oilseed rape indicates pollinators play an important role in the crop's development and yield. With this in mind, Picard-Nizou *et al.* (1995) attempted to determine if genetically modified oilseed rape had an effect on the foraging behavior of bees. Two transformed oilseed rape lines carrying a gene for the expression of chitinase in somatic tissues to enhance disease resistance and two untransformed lines were used to evaluate the foraging behavior of bees. Experiments were conducted in controlled indoor flight rooms and outdoor flight cages. The researchers monitored general bee behavior (total number of visits) and individual bee behavior using a video camera with special software. Because the bees forage oilseed rape for nectar, both the transformed and untransformed lines were analyzed for nectar quality. The results indicated the transformed plants did not induce any significant change to bee foraging behavior.

Although oilseed rape is a self-fertile crop it appears an external agent is necessary to transfer pollen from the anthers to the stigma. Many observations and experiments have been completed but the role of pollinators remains unclear. Movement of plants by wind probably increases self-pollination and wind transport of pollen probably increases cross-pollination. However, wind is an unpredictable pollination agent and it is difficult to assess. Oilseed rape has

the morphologic, anatomic, and physiologic characteristics of an insect pollinated flower. Insects are considered to be important and effective pollinators of oilseed rape and are required for effective cross-pollination.

2.2 TRANSGENIC HERBICIDE RESISTANT OILSEED RAPE

Herbicides have become an important method of weed control in modern agriculture. They provide economically superior weed control and are more labor and energy efficient than manual or mechanical cultivation methods (Stalker *et al.* 1996). The mode of action of many herbicides is to inactivate target proteins essential for vital plant functions like photosynthesis. Since crop plants and weeds share similar plant functions, only nonselective herbicides can be used without damage to the crop. An alternative is to confer resistance in crops to broad-spectrum herbicides.

Gene transfer techniques have been developed to incorporate an enzyme or enzyme system into plants that will degrade or detoxify the herbicide before it reaches the target site of action or modify a plant enzyme or biochemical target to render it insensitive to the herbicide or to induce the overproduction of the unmodified target protein permitting normal metabolism to occur (Stalker *et al.* 1996).

The result of the gene transfer technique is a transgenic herbicide resistant crop, which is able to withstand the application of a broad spectrum herbicide due to

the presence of a transgene. A transgenic plant has genetic material that has been altered in a way that does not naturally occur by mating and/or natural hybridization (Noome 1995). Transgenes are the gene(s) inserted in the plant from a foreign species usually conferring a novel trait like herbicide resistance (Crawley *et al.* 1993).

2.2.1 Bromoxynil Herbicide Resistant Oilseed Rape

Bromoxynil is a photosynthetic inhibitor that blocks the flow of electrons by binding to a component of the quinone-binding protein complex of the photosynthetic electron transport chain (Stalker *et al.* 1988; Duke 1990; Holt *et al.* 1993). This blockage results in the destruction of the photosystem II reaction center in broadleaf plants (Gronwald 1994). Chemically grouped as a hydroxybenzonitrile, bromoxynil is used to control dicotyledonous weeds in monocotyledonous/cereal crops (Freyssinet *et al.* 1996). Monocotyledons possess a nitrilase gene conferring the ability to metabolize bromoxynil herbicides into non-phytotoxic benzoic acid forms (Stalker *et al.* 1996).

A nitrilase gene capable of metabolizing bromoxynil into a nontoxic metabolite, 3,5-dibromo-4-hydroxybenzoic acid, was cloned from *Klebsiella pneumoniae* subsp. *ozanae* (Stalker and McBride 1987; Stalker *et al.* 1988). This soil bacterium was isolated from a site contaminated with bromoxynil and introduced into oilseed rape via *Agrobacterium tumefaciens* mediated transformation system. Two constructs designated 235 and 237, were developed when

researchers with Rhône-Poulenc, France introduced the nitrilase gene into oilseed rape. For construct 235, the coding region of the nitrilase gene was placed under the control of a constitutive promoter (CaMV), while for construct 237, a light inducible promoter (RuBisCO) was used (Freyssinet *et al.* 1996). The gene encoding bromoxynil resistance is inherited in a Mendelian fashion and exhibits dominance when the plants are sprayed with bromoxynil (Stalker *et al.* 1996). Transformed oilseed rape varieties are able to withstand several times the normal field dosage for broadleaf weed control in cereals (McVetty, unpubl.).

2.3 BIOLOGICAL COST

Genetic crop improvement includes the introduction of new gene(s) in a crop to improve productivity or resist pests. Researchers believe the novel herbicide resistant transgenics could effect the phenotype and/or performance of the crop (including seed quality traits) under field conditions by disrupting some essential metabolic functions or diverting energy and limited resources to support pathways associated with herbicide resistance (Hails *et al.* 1997; Bergelson *et al.* 1996; Mallory-Smith and Eberlein 1996; Arnoldo *et al.* 1992). Plant injury can also occur if the herbicide resistance is incomplete. These negative effects on crop phenotype or performance are referred to as biological cost.

A comparison including agronomic parameters like days to flower, days to maturity, seed yield, oil content, protein content, and associated quality traits

between isogenic lines or isogenic populations will reveal if a biological cost is associated with the new trait.

Three processes could affect the performance of transgenic plants: Insertional mutagenesis. Insertional mutagenesis is a transformation process which introduces DNA to disrupt the function of the plant gene it is inserted in or near (Dale and McPartlan 1992; Eberlein *et al.* 1998). Pleiotrophy. Individual genes can have apparently unrelated, multiple effects on plant phenotype (Boerboom and Lauer 1997; Dale and McPartlan 1992). Somaclonal variation. The production of transgenic plants involves a tissue culture selection and regeneration phase known to cause genetic variation among regenerated plants (Dale and McPartlan 1992; Eberlein *et al.* 1998). With herbicide resistance, additional biological costs could arise from excessive enzyme production or less than complete herbicide resistance resulting in plant injury.

Previous studies on both herbicide resistant weeds and crops indicate a biological cost is sometimes associated with the resistance trait (Gressel and Ben-Sinai 1985; Beversdorf *et al.* 1988).

Any significant biological cost resulting from crop improvement needs to be detected and characterized. To this end, researchers have studied a number of crop plants to determine if a biological cost related to herbicide resistant genes exists. In order to evaluate the pleiotropic effects of herbicide resistance, it is

essential that comparisons are made between resistant and susceptible isogenic lines or isogenic populations that differ only in the resistance trait. Results from studies where isogenic genotypes were not used should be considered with great caution.

2.3.1 Transgenic Crops

2.3.1.1 Potato - *Solanum tuberosum*

Dale and McPartlan (1992) compared the field performance of potato plants derived from three sources: transgenic plants derived from co-cultivation of tuber discs transformed with *Agrobacterium tumefaciens*, plants regenerated from tuber discs without transformation, and plants established from tuber nodal shoot cuttings. The purpose of this experiment was to separate the effects of the tissue culture process (tuber discs and shoot cuttings) and the inserted genes on plant performance. The transgenic potatoes contained two genes, a reporter gene for betaglucuronidase (GUS) and the neomycin phosphotransferase (NPTII) gene.

Plant performance was measured in terms of plant height at flowering, weight of tubers, number of tubers, weight of large tubers, and the number of larger tubers. Results indicated the tissue culture process and the inserted genes had a significant effect on the field performance of potatoes. Shoot cultured plants performed significantly better than tuber disc regenerants for all the parameters measured. The difference between the nontransgenic and transgenic isolines was significant. The nontransgenic plants produced taller, higher yielding, and

heavier tubers than the transgenic lines. When the transgenic lines were compared, the researchers observed significant differences indicating the inserted genes had an impact on the plants' performance. They determined the NPTII gene had little, if any effect on performance unlike the GUS gene, which had a marked effect on plant performance.

De Greef *et al.* (1989) evaluated four isogenic herbicide resistant potato lines under field conditions. The transgenic potatoes contained the phosphinothricin acetyl transferase gene (PAT) conferring resistance to glufosinate ammonium herbicide. They determined there were no significant differences between the unsprayed controls and the sprayed transformed lines. The sprayed transgenic lines consistently yielded more than the controls at the lower (1000 g a.i/ha) herbicide dosage. When the dosage was increased to 4000 g a.i./ha (four times the recommended field rate), the tuber yields of the sprayed transgenics were lower than the unsprayed controls. The potato plants were slightly discolored from the increased rate of herbicide, but no plants were killed. The researchers concluded the growth of the transgenic lines was indistinguishable from the nontransgenic controls indicating there is no biological cost associated with the inclusion of the PAT gene in potato when the recommended rate of herbicide is applied. However, if a higher rate of glufosinate ammonium is applied to achieve greater weed control, a reduction in yield will occur. This demonstrates that at increased herbicide rates, a biological cost is associated with the incorporation of the PAT gene in potatoes.

Tuber yield and quality in four transgenic bromoxynil resistant isogenic potato lines were compared to nontransgenic potatoes under field conditions (Eberlein *et al.* 1998). Both lines were evaluated with and without herbicide to determine the effect of herbicide on yield. Bromoxynil killed a large portion of the nontransgenic, control plots. Tuber quality was assessed by measuring specific gravity and percent solids of the harvested tubers. Fry color was also noted for all samples. In the yield trials, the best performing transgenics had total tuber yields equal to the untreated control but the U.S. No. 1 tuber yields were 15 to 30% lower than the untreated controls. Tubers produced by three of the four lines has specific gravities, percent solids, and fry color similar or better than the untreated control. The researchers believe the difference in tuber yield was due to somaclonal variation rather than the bromoxynil resistant gene since three other studies involving three transgenes other than bromoxynil resistance, produced similar results.

2.3.1.2 Flax - *Linum usitatissium*

The agronomic performance of two transgenic herbicide resistant flax lines was evaluated against their nontransgenic parent (McHughen and Holm 1991). The transgenic flax contained a mutant *Arabidopsis* ALS gene, which confers resistance to the soil applied sulfonylurea herbicides. Herbicide treated plots were compared to untreated plots and the parental, nontransgenic control. Agronomic parameters measured included height, plot biomass, seed yield, and 1000 seed weight. There were no significant differences between the transgenic

lines and the parent for any agronomic trait measured in the untreated soil indicating there is no detrimental effect of the ALS gene. There were also no significant differences for the transgenic lines between the untreated and the herbicide treated soils.

2.3.1.3 Corn - *Zea mays*

The agronomic performance of imazethapyr resistant corn was compared with susceptible isogenic lines (Boerboom and Lauer 1997). Imazethapyr resistant corn was developed through mutation breeding and tissue culture selection and is not considered a transgenic. Resistance to acetolactate synthase (ALS) inhibiting herbicides is provided by one of three genes. 10 isogenic hybrid pairs were compared under weed free conditions and without imazethapyr to determine if yield differences occurred. The agronomic parameters measured were yield, grain moisture, and stalk strength. When evaluated over eight environments, only one resistant hybrid yielded less than its susceptible hybrids. The other 9 resistant hybrids had yields equal to or greater than their susceptible hybrids. No differences were detected in grain moisture and stalk strength. This data indicates a yield penalty or other negative effects on agronomic characteristics are not associated with the imazethapyr resistance trait in corn and therefore, there is no biological cost of this herbicide resistance in this crop. Imidazolinone herbicides bind to the acetolactate synthase (ALS) enzyme and block the production of the branched chain amino acids, isoleucine, leucine, and valine in plants. By modifying this binding site, researchers have been able to

engineer resistance to imidazolinone herbicides. The modification allows the ALS enzyme to convert pyruvate to α-acetolactate, one of the steps necessary to produce the branched-chain amino acids. The rate at which this conversion takes place is referred to as ALS activity. Researchers have observed the rate of ALS activity in imidazolinone resistant lines is half to two thirds the rate of the imidazolinone susceptible lines (Mallory-Smith and Eberlein 1996). This appears to be the only detectable pleiotrophic effect observed in imidazolinone resistant corn.

Sethoxydim resistant conventional corn isogenic lines were tested under field conditions by Dotray *et al.* (1993). No differences were detected in yield, plant height, days to 50% silking, and rate of kernel drying between the resistant and susceptible plants. The resistance gene also did not affect germination of the crop or seedling vigor. Therefore, no biological cost is associated with this herbicide resistance in corn.

2.3.1.4 Oilseed Rape - *Brassica napus*

2.3.1.4.1 Triazine Resistant Oilseed Rape

Triazine is a photosynthetic inhibiting herbicide that interferes with the photosynthetic electron transport chain (Duke 1990). Resistance is due to a point mutation in the *psb A* chloroplast gene, which codes for a 32 kDa protein resulting in the substitution of glycine for serine (Mallory-Smith and Eberlein

1996). The mutation prevents the herbicide from binding but it also results in reduced photosynthetic productivity.

Triazine resistant oilseed rape was the first broadleaf herbicide resistant crop available to producers. Using traditional breeding techniques, plant breeders transferred the naturally occurring resistance from *Brassica rapa* to oilseed rape (Beversdorf and Kott 1987). Triazine resistance is cytoplasmically or maternally inherited therefore the progeny of a resistant individual used as a female are resistant. The presence of the resistant cytoplasm in oilseed rape results in a 20 to 30% yield penalty compared to isogenic lines in the susceptible cytoplasm (Beversdorf and Kott 1987; Holt *et al.* 1993; Beversdorf *et al.* 1988; Gronwald 1994; Grant and Beversdorf 1985; Holt and Thill 1994). Beversdorf *et al.* (1988) also observed triazine resistant oilseed rape flowered an average of two days later and were shorter at maturity than isogenic triazine susceptible oilseed rape. The production of triazine resistant varieties was common in areas where the pressure from related weeds like wild mustard was high (Gronwald 1994). Otherwise, the biological cost was too high for cost effective use of this herbicide resistance.

Isogenic lines of triazine resistant and triazine susceptible oilseed rape were evaluated in a competition study. Gressel and Ben-Sinai (1985) observed a decrease in fresh and dry weight, in the number and weight of the seed pods, and in seed yield of the resistant plants when compared to the susceptible plants.

2.3.1.4.2 Kanamycin Resistance

Arnoldo *et al.* (1992) compared 8 transgenic isogenic populations of the oilseed rape cultivar Westar to Westar and compared 3 transgenic isogenic populations of the oilseed rape cultivar Regent to Regent under field conditions to assess the impact of introduced foreign genes, as well as the tissue culture manipulations required to transform the plants. The transgenic oilseed rape lines carried a neomycin phosphotransferase (NPTII) gene for kanamycin resistance. The agronomic parameters evaluated included maturity, yield, and oil and protein content. No differences were detected in maturity between the transgenic Westar and Regent isogenic populations and nontransgenic Westar and Regent populations. The yield per transgenic plant (measured in grams) was in most cases higher than the nontransgenic plants, however, the difference was not significant. No statistical differences were detected for percent oil and percent protein. The oil and protein content profiles were typical for oilseed rape. These results indicate oilseed rape can be successfully genetically engineered and the *Agrobacterium* based transformation system does not induce any adverse effects on the important agronomic and qualitative traits for canola. There appears to be no apparent biological cost associated with the antibiotic resistance gene in this crop.

Kemble *et al.* (1991) studied the performance of kanamycin resistance in isogenic oilseed rape populations over a two year period under field conditions.

No deleterious effects were found in either agronomic (maturity and yield) or quality (oil and protein content) parameters.

2.3.1.4.3 Glufosinate Ammonium Resistance – Liberty Link Oilseed Rape

Glufosinate ammonium resistance oilseed rape has been under field evaluation in Canada since 1989. Field trial data from 1989 and 1990 indicated the isogenic resistant line was capable of withstanding commercial application rates of glufosinate herbicide. The transgenic population was similar to the control in agronomic performance and most quality characteristics (Beversdorf 1992). The fatty acid composition of the transgenic line was canola quality. Oelck *et al.* (1991) completed a similar study where isogenic lines were compared at only one location in Saskatchewan during the 1990 growing season. This study considered the effect of the PAT gene on oil quality characteristics. The results of the trial indicate the derived transgenic line's oil content was lower than the nontransgenic population. Even though the data is preliminary, it appears a biological cost could be associated with glufosinate ammonium resistance in oilseed rape.

Kumar *et al.* (1998) evaluated the agronomic performance and seed quality traits of nineteen unsprayed glufosinate ammonium tolerant and susceptible isogenic pairs at two sites in Saskatchewan in a two year study. Each tolerant line was produced from a single transformation event and contained either one or two inserts of the PAT gene. Several lines had delayed flowering and maturity, lower

biomass and seed yield, and lower oil and protein content. The four lines with two inserts of the PAT gene were more negatively affected than the fifteen tolerant lines with one insert. Six tolerant lines were similar in agronomic performance and seed quality traits than their susceptible isogenic pair, no negative affects were observed. These results indicate that there is a positional effect of the PAT gene on the performance of some tolerant lines, however it is possible to generate tolerant lines without any negative affects on the growth, development, and productivity of oilseed rape.

2.4 OILSEED RAPE POLLEN MEDIATED GENE FLOW

The outcrossing rate of oilseed rape for important agronomic and quality traits in the field is presumed to be minimal and for the most part has been ignored by canola breeders. A number of qualitative (elevated erucic acid levels) and quantitative (petal color, stem color, and isozyme polymorphisms) markers have been used to determine the rate of outcrossing in oilseed rape.

Olsson (1960) monitored petal color to determine the outcrossing rate in spring and winter oilseed rape lines in Svalöf, Sweden. Yellow petal color is controlled by a single, nuclear gene and is dominant to white petal color. A white petal oilseed rape plant was completely surrounded by yellow petal oilseed rape plants. At maturity, the white petal plants were harvested and the seeds planted to evaluate petal color. Outcrossing was determined by dividing the number of yellow petal plants by the total number of plants evaluated. The average

interplant outcrossing rate was 36% in spring oilseed rape and 34.6% in winter oilseed rape. Persson (1956 as cited by Lewis and Woods 1991) completed a similar study also in Sweden using petal color as a marker. He determined the average interplant outcrossing rate in spring oilseed rape was 27 to 30%. Further studies indicated the results of these studies are biased since honeybees are sensitive to flower color (Lewis and Woods 1991).

Researchers investigating outcrossing in oilseed rape using elevated erucic acid levels as a marker, reported outcrossing rates ranging from 5% to 75% (Rakow and Woods 1987; Lewis and Woods 1991; Hühn and Rakow 1979). Hühn and Rakow (1979) studied five high erucic acid and five low erucic acid varieties grown under field conditions in northern Germany to determine the rate of outcrossing between plots of winter oilseed rape. They estimated the interplot outcrossing rate ranged from 2.8 to 7.5%. Rakow and Woods (1987) also used erucic acid as a marker to monitor outcrossing rates in five lines of spring oilseed rape over a three year period in Saskatchewan. Low erucic acid plants were planted between rows of high erucic acid lines spaced 1.5 m. The low erucic acid plants were harvested individually and assessed for erucic acid content. In total 3400 seeds were screened from 92 plants. The interplant outcrossing rates of each line ranged from 17.9 (±3.5) to 27.5% (±2.8) with an average of 21.8% (±3.0). Outcrossing rates for individual plants varied between 2 and 75%. Lewis and Woods (1991) also monitored interplant outcrossing in spring oilseed rape using elevated erucic acid levels. Over a two year period in Alberta they

screened 5700 seeds produced by 278 plants. They estimated the average interplant outcrossing rate to be 50%.

Gowers (1981) used stem color to determine the rate outcrossing between oilseed rape plants in Scotland. Purple stem color (anthocyanin) is controlled by a single, nuclear gene and is dominant to green stem color. Four green stemmed oilseed rape cultivars and one purple stemmed cultivar were evaluated. Each green stem plant was completely surrounded by 24 purple stemmed plants. 250 seedlings of each cultivar were evaluated for the presence or absence purple stem to determine the average interplant outcrossing rate. The average outcrossing rate for three cultivars was 18%, but the fourth cultivar averaged 43%. Outcrossing rates of individual plants ranged from 4% to 80%. Pollen germination and pollen tube penetration tests revealed the progeny of the fourth cultivar have high levels of self-incompatibility. Since the pollen produced on the incompatible plants cannot successfully fertilize the plant's ovules, the seeds produced are therefore the result of an outcrossing event.

The outcrossing rate obtained when self-fertile and self-sterile lines are studied provides researchers with a theoretical maximum level of outcrossing because there is no pollen competition between the two lines. This maximum level is important to breeders of hybrid crops where they require outcrossing to reach 100%. However, in a conventional breeding program the upper limit of

outcrossing is of little significance. A representative rate of outcrossing can only be obtained if two self-fertile lines are studied.

Becker *et al.* (1992) investigated interplant outcrossing rates by isozyme electrophoresis. He studied the patterns of 3 isozymes: diaphorase (DIA), glucosephosphate isomerase (GPI), and shikimate dehydrogenase (SDH) by comparing the pattern of the mother plants with their progeny. 200 to 300 plants were selected from the field and screened for polymorphisms, those with rare patterns were harvested and their seedlings analyzed. Interplant outcrossing ranged from 12 (±8.6) to 47% (±10.0) with an average of 34% (±11.7). These results are based on an average of only eight plants.

They also observed the outcrossing rate was greatly influenced by the position of the flower on the plant. The lower portion of the plant had the highest rates (46%) and the top of the plant had the lowest rates (10%). Since flowering starts with the lowest flower on the main raceme and ends with the flowers at the top of the side branches, the authors believe there are two possible explanations for the influence of flower position on outcrossing. This effect could be the result of either an accidental change in climatic conditions towards the end of flowering period or there may be a general tendency for outcrossing rates to change during flowering. However, they were unable to be specific. It is also possible the flowering period of the lines studied was not synchronous. If one line stopped flowering before the other, the outcrossing rates would be greatly influenced.

The authors did not comment on flower synchrony so we are unable to determine if the outcrossing rates observed in this study were influenced by the respective flowering periods of the two cultivars used in the study.

Becker *et al.* (1992) also considered the effect of five different geographical locations on outcrossing rates. The trials were conducted at three sites in Sweden (Kölbäck, Svalöv, and Landskrona), one site in Denmark (Dyngby), and one site in northern Germany (Natendorf). The outcrossing rate in the most northern location, Kölbäck, was only 12% (±8.6) compared to the other locations, which ranged between 32% (±20.4) to 47% (±10.0). The outcrossing rate in Kölbäck was significantly different from the other locations but the other locations did not significantly differ from each other. Based on the results of this study and the other studies mentioned above, it appears location has an affect on the outcrossing rate of oilseed rape.

Pollen flow between oilseed rape cultivars can greatly influence the final quality of a oilseed rape crop. Two separate studies by Bilsborrow *et al.* (1994; 1998) considered the possibility of contaminating oilseed rape crops with the pollen from high erucic acid rapeseed (HEAR) varieties grown nearby. Depending on the level of pollen contamination, the oilseed rape seedlot could be rejected. Bilsborrow *et al.* (1994) reported the concentration of pollen decreased rapidly with distance from the edge of the plot and within 2 m the concentration was reduced by 50%. When oilseed rape samples were evaluated for erucic acid

content, they found low levels of contamination. Some variation was observed in levels but this was attributed to insect activity rather than wind mediated pollen transfer. The authors concluded wind plays an important role in oilseed rape pollination by moving the plants together (mechanical pollination) which permits pollen transfer to occur but it does not carry the heavy and sticky pollen grains any significant distance. Similar results were obtained in another study by Bilsborrow *et al.* (1998). The level of pollen contamination was generally low and random throughout the oilseed rape blocks. Because HEAR pollen concentrations decreased rapidly with distance from the oilseed rape blocks this suggests that contamination between edible oilseed rape and high erucic acid oilseed rape does not present a major problem under field scale cultivation.

Transgenic herbicide plants provide researchers with a unique opportunity to measure outcrossing. Cresswell (1994) used transgenic oilseed rape to quantify outcrossing that results from a single pollinator visit. The transgenic line was homozygous dominant for the bar gene, which confers resistance to glufosinate ammonium herbicide. Bumble bees mediated gene flow in a three-chambered cage. The first chamber contained four to six nontransgenic plants, a single transgenic pollen donor was placed in the second chamber, and four to six nontransgenic pollen recipients were contained in the third chamber. In each of four trials, several bumble bees entered the first chamber where they were allowed to visit about 20 flowers before one bumble bee was permitted to pass to the second chamber. After the bumblebee had visited 5 flowers on the

transgenic plant it was allowed to pass to the third chamber. All the open flowers in the third chamber were uniquely marked with a colored paint on the pedicel so the bumblebee's floral visits could be recorded. All visits were noted until revisits became consistent. In total 37 flowers were visited in the third chamber.

The seeds produced by the 37 flowers pollinated in the third chamber were harvested and their seedlings were sprayed with herbicide to determine outcrossing. Of the 691 seeds produced only 32 or 5% survived the herbicide application. When the pattern of pollen dispersal was examined 91% of the resistant seedlings resulted from the first four flowers pollinated. No resistant individuals were detected after the fourteenth successive flower visited which indicates the resistant pollen was replaced fairly rapidly with susceptible pollen as the pollinator continued to forage. He did not, however, provide any information on the foraging behavior of the bumblebees. It is important to know whether the bees moved from plant to plant or if they moved from flower to flower. If they moved from plant to plant and if the neighboring plants were transgenic the results of this could be very different.

Oilseed rape outcrossing rates vary depending on the marker, location, and design of the experiment used. The results of these studies indicate oilseed rape readily outcrosses within its species, which could lead to the contamination of breeding lines or unexpected genetic segregation results in a field-based oilseed rape plant breeding program.

3.0 A STUDY OF THE BIOLOGICAL COST OF TRANSGENIC BROMOXYNIL RESISTANCE IN OILSEED RAPE

3.1 INTRODUCTION

Bromoxynil resistance in oilseed rape (*Brassica napus)* is conferred by a single nitrilase gene cloned from the soil bacterium *Klebsiella pneumoniae* subsp. *ozanae* that is capable of metabolizing hydroxybenzonitrile herbicides. The soil bacterium was isolated from a site contaminated with bromoxynil and introduced in oilseed rape via an *Agrobacterium tumefacians* mediated transformation system. Two constructs, 235 and 237, were developed when the nitrilase gene was introduced into oilseed rape (Freyssinet *et al.* 1996). The transformed varieties of oilseed rape are able to resist an application of bromoxynil herbicide several times the normal field rate (280 g a.i./ha) for broadleaf weed control in cereals (McVetty, unpubl.).

Many previous studies on both herbicide resistant weeds and crops have indicated that a biological cost is associated with herbicide resistance. This is especially so for triazine resistance (Gressel and Ben-Sinai 1985; Beversdorf *et al.* 1988). The biological cost of adding bromoxynil resistance to oilseed rape has not yet been studied, but is of substantial scientific interest. Oilseed rape may be the ideal candidate for a biological cost study, since a wealth of knowledge and experience exists with regard to breeding varieties of oilseed

rape exists. Isogenic lines and populations of summer rape with canola quality, differing only in the bromoxynil resistance trait, can be generated and various agronomic and quality characteristics of these lines can be compared under field conditions.

The objective of this research is to compare the agronomic performance and seed quality traits of isogenic oilseed rape lines and populations to determine if a biological cost is associated with the addition of bromoxynil resistance in oilseed rape.

3.2 MATERIALS AND METHODS

3.2.1 Biological Cost Field Trials

Biological cost field trials were established at the University of Manitoba Research Farm at Carman (hereafter "Carman") 1994 through 1997, at the Integrated Crop Management Systems Research Farm at Portage la Prairie (hereafter "Portage") 1995 through 1997, and at the University of Manitoba at Winnipeg (hereafter "Winnipeg") 1994 through 1997.

The entries used in the biological field trials included 235 245 (the original single transformed line derived by transformation of a single plant of Westar), Westar 235 (W 235 - a pure breeding bromoxynil resistant F_4 isogenic population of Westar), Westar 237 (W 237 - a pure breeding bromoxynil resistant F_4 isogenic population of Westar), and Westar (an open pollinated population cultivar). W 235 and W 237 originated from crosses conducted at the University of Manitoba, while 235 245 was developed by Rhône-Poulenc.

The trial was arranged as a randomized complete block design with four replicates. In 1994 and 1995, the four randomized entries included Westar, W 235, W 237, and 235 245. The resistant plots did not receive an application of bromoxynil in 1994 or 1995. In 1996, a sprayed treatment for the herbicide resistant entries W 235, W 237, and 235 245 was included in the trials to determine if any differences existed between the unsprayed and sprayed plots of

the resistant entries for the characters measured. In 1997, the five randomized treatments were Westar unsprayed, 235 245 unsprayed, 235 245 sprayed, W 235 unsprayed, and W 235 sprayed. W 237 was eliminated from the biological cost trials in 1997.

A small plot belt cone seeder was used to seed the plots at a rate of 8 kg/ha. Carbofuran (10% granules) insecticide was banded with the seed at a rate of 12 kg a.i./ha to control flea beetles (*Phyllotreta cruciferae* Goeze and *P. striolata* F.). Individual plot size was 3 m long and 1.2 m wide and consisted of six rows of canola on a 20 cm row spacing. The trials were seeded over eight ranges or blocks with a 1.5 m pathway between the ranges. The Carman trial was seeded on May 30,1994, May 24,1995, May 23,1996, and May 15, 1997. The Portage trial was seeded on May 30, 1995, May 28, 1996, and May 26, 1997. The Winnipeg trial was seeded on May 18, 1994, May 29 in 1995, May 30, 1996, and May 27, 1997.

Bromoxynil was applied on the sprayed plots at 280 g a.i/ha (the recommended rate for cereals) when the oilseed rape plants reached the two to four leaf stage approximately 21 days after seeding. The plots were sprayed in Carman on June 13, 1996 and June 6, 1997, in Portage on June 17, 1996 and June 11, 1997, and in Winnipeg on June 17, 1996 and June 16, 1997. Bromoxynil was applied using a bicycle wheel plot sprayer equipped with flat fan nozzles delivering 108 L/ha at 275 KPa. The sprayed plots were visually assessed three

and seven days after treatment using the Expert Committee on Weeds (ECW Western Canada Section) 0 to 9 rating scale to evaluate crop herbicide tolerance. A rating of zero indicates complete crop kill, while a rate of nine indicates complete crop tolerance. A score of seven or better is considered commercially acceptable. Herbicide resistant ratings after 3 and 7 days were 9 for all resistant plots therefore crop resistance scores are not presented in any of the data tables. Crop showed complete tolerance.

A small plot swather and combine were used to harvest the individual plots. A subsample of the harvested seed from each plot was assessed for oil and protein content.

3.2.2 Crop Measurements

Several agronomic parameters were assessed for each treatment/entry during the growing season including flowering, plant height, lodging, maturity, and yield.

Flowering was assessed as days after sowing to 50% of the plants showing at least one flower open. Plant height was measured in centimeters after pod set. Lodging was assessed prior to maturity using a 1 to 5 scale, where 1 is completely standing and 5 is completely flat. Maturity was assessed as days after sowing to physiological maturity. Seed yield was recorded as g/plot and then converted to kg/ha. Seed oil content and seed protein content were measured and reported as a percentage of the seed at 0% moisture. Seed oil

content was measured using Nuclear Magnetic Resonance, while protein content was determined using the LECO combustion method. Erucic acid content and seed glucosinolates were measured on one of the four replicates using gas chromatography. Erucic acid is expressed as a percentage of the fatty acids in the oil. Glucosinolates were expressed in micromoles glucosinolates per gram of oil-free meal at 8.5% moisture.

3.2.3 Statistical Analysis

Data for this experiment was analyzed using analysis of variance techniques. Due to differences in the number of years the trials were conducted at each site, data from each site-year was expressed as an environment. Single degree of freedom orthogonal contrasts were used to statistically compare the relative performance of the respective transgenic herbicide resistant populations and line to the nontransgenic line, and the respective sprayed resistant populations and line to respective unsprayed resistant populations and line. The contrasts were tested for significance using the residual error term. Significant differences were identified using a LSD test (5% level). All statistical analyses were performed on SAS System for Windows v 6.12 (SAS Institute Inc. Cary, North Carolina, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Biological Cost Trials

At all sites (environments), canola stands and growth were normal and visually comparable to 'good' commercial field crops. Flowering was extended by approximately one week at Winnipeg in 1996, due to regrowth following a hailstorm on July 16. There were no other apparent detrimental effects of the hailstorm on other characteristics measured including yield.

3.3.2 Comparisons within Genotype for Effect of Spraying

The performance of two bromoxynil resistant transgenic populations, Westar 235, Westar 237, and one transgenic line, 235 245, when sprayed with bromoxynil, were compared to the performance of these respective genotypes unsprayed. While the enzyme that breaks down bromoxynil (nitrilase) is present within the plant whether the herbicide is applied or not, the application of bromoxynil actually challenges the activity of this enzyme and effectiveness of the herbicide resistance. There was only one significant difference in the measured characteristics between sprayed and unsprayed for all three genotypes. This was for 235 245 for yield, where the mean of the unsprayed plots was significantly higher than for the sprayed plots (Table 3.1). Since there were no other significant differences detected for the other genotypes and characteristics between sprayed and unsprayed it is doubtful that this yield depression was due to the application of bromoxynil herbicide.

Table 3.1. Effect of spraying bromoxynil herbicide (280 g a.i./ha) on lodging, plant height, days to flower, days to maturity, yield, oil content, protein content, and sum of oil and protein content means for two transgenic bromoxynil resistant *B. napus* populations and one transgenic bromoxynil resistant *B. napus* line, 1996 to 1997

Genotype	Lodging (1-5)	Height (cm)	Days to Flower	Days to Maturity	Yield (kg/ha)	Oil (%)	Protein (%)	Sum[3] (%)	Station-Years
W 235 U[1]	2.9	123	42	85	2675	45.6	24.9	70.5	6
W 235 S[2]	3.2	122	43	86	2525	45.6	24.9	70.5	6
LSD (0.05)	0.4	4.6	1.1	1.1	164	0.8	0.8	0.3	
W 237 U	3.7	126	41	86	2804	45.2	25.4	70.6	3
W 237 S	3.5	129	41	86	2692	45.4	24.6	70.0	3
LSD (0.05)	0.3	5.8	0.3	0.9	303	0.8	1.3	0.8	
235 245 U	3.7	120	41	85	2706	45	25.4	70.5	6
235 235 S	3.2	120	41	85	2384*	45.6	25	70.6	6
LSD (0.05)	0.6	4.5	0.4	0.5	168	0.7	0.8	0.3	

*Indicates significance at the 5% level for within genotype comparisons

[1]U=unsprayed

[2]S=sprayed

[3]Sum=sum of oil and protein content

Based on these results, these bromoxynil resistant oilseed rape lines do not appear to experience any damage when they are sprayed with the recommended rate of bromoxynil as compared to when these genotypes are unsprayed. A study of glufosinate transgenic herbicide resistant potatoes also concluded that a spray effect was not associated with the phosphinothricin acetyl transferase (PAT) gene when the crop was sprayed with the recommended rate of glufosinate ammonium (De Greef *et al.* 1989). However, when the rate of herbicide was increased, a spray effect was observed. The transgenic lines of potatoes were injured and produced significantly lower yields than the nontransgenic potato unsprayed control. In a separate study by McVetty (unpubl.), Westar 235 and Westar 237 were able to withstand several times the recommended rate of bromoxynil without crop injury and without a loss in yield.

3.3.3 Comparisons of Transgenic Bromoxynil Resistant Isogenic Populations and a Transgenic Bromoxynil Resistant Line to Westar

The performance of the bromoxynil resistant isogenic populations and line (Westar 235, Westar 237, and 235 245) compared to Westar revealed few significant differences for the characteristics measured (Tables 3.2). The most important comparisons are Westar 235 versus Westar and Westar 237 versus Westar. The isogenic line 235 245 versus Westar is included for general interest, however, this comparison is confounded since 235 245 is a single genotype line derived from a population of Westar. A single genotype line selected at random

Table 3.2. Transgenic bromoxynil resistant isogenic populations and isogenic line compared to nontransgenic Westar (population) for lodging, plant height, days to flowering, days to maturity, yield, oil content, protein content, and sum of oil and protein content means, 1994 to 1997

Genotype	Lodging (1-5)	Height (cm)	Days to Flower	Days to Maturity	Yield (kg/ha)	Oil (%)	Protein (%)	Sum[3] (%)	Station-Years
Westar	3.2	122	43	87	2258	45.6	25.0	70.6	11
W 235 U[1]	3.0	127*	44*	87	2389*	45.5	25.4	70.8*	11
LSD (0.05)	0.4	2.9	0.4	0.4	113	0.4	0.5	0.2	
Westar	3.3	116	42	85	2560	45.9	24.5	70.4	6
W 235 S[2]	3.2	122*	43*	86*	2525	45.6	24.9	70.5	6
LSD (0.05)	0.3	3.9	0.4	0.5	153	0.7	0.7	0.4	
Westar	3.2	129	43	88	2237	45.4	25.3	70.7	8
W 237 U	3.5	129	44*	88	2211	45.0	25.9*	71.0*	8
LSD (0.05)	0.4	3.9	0.6	0.4	136	0.5	0.6	0.3	
Westar	3.6	125	41	86	2805	45.8	24.7	70.4	3
W 237 S	3.5	129	41	86	2692	45.4	24.6	70.0	3
LSD (0.05)	0.4	6.4	0.8	0.8	207	0.7	0.7	0.8	
Westar	3.2	122	43	87	2258	45.6	25.0	70.6	11
235 245 U	3.5	124	43	87	2305	45.0*	25.6*	70.6	11
LSD (0.05)	0.4	2.9	0.4	0.4	113	0.4	0.5	0.2	
Westar	3.3	116	42	85	2560	45.9	24.5	70.4	6
235 245 S	3.2	120*	41*	85	2384*	45.6	25.0	70.6	6
LSD (0.05)	0.3	3.9	0.4	0.5	153	0.7	0.7	0.4	

*Indicates significance at the 5% level for within genotype and within sprayed or unsprayed comparisons to Westar

[1]U=unsprayed

[2]S=sprayed

[3]Sum=sum of oil and protein content

from a population could perform equal to, better than, or worse than its source population for any given trait.

3.3.3.1 Westar 235 versus Westar – Isogenic Population Comparison

Westar 235 unsprayed was significantly different from Westar only for height (5 cm taller), days to flower (1 day later), yield (6% higher) and sum of oil and protein content (0.2% higher) (Table 3.2). It is interesting to note that the transgenic herbicide resistance in *B. napus* appears to have a positive effect on yield and sum of oil and protein content, even in the absence of the herbicide.

When Westar 235 sprayed was compared to Westar, the only significant differences were height (6 cm taller), days to flower (1 day later) and days to maturity (1 day later) (Table 3.2). For Westar 235 sprayed, there were no significant differences in yield and oil or protein content characteristics which indicated the one day delay to flower and maturity did not significantly affect the performance of Westar 235 sprayed. These few, small differences between 235 unsprayed/sprayed and Westar indicate that there is little or no biological cost associated with the 235 bromoxynil resistance construct in oilseed rape.

3.3.3.2 Westar 237 versus Westar – Isogenic Population Comparison

Westar 237 unsprayed was significantly different from Westar only for days to flower (1 day later), protein content (0.6% higher), and sum of oil and protein content (0.3% higher) (Table 3.2). The positive effect for protein content and

sum of oil and protein content, along with no significant differences for yield, indicated that the one day delay in flowering had minimal effect on the performance of the isogenic population

When Westar 237 sprayed was compared to Westar, no significant differences were obtained for any of the characteristics measured (Table 3.2). The inclusion of the bromoxynil resistance gene in the 237 construct appears to have no effect on the performance of the transgenic population and therefore indicates that there is no biological cost associated with the bromoxynil resistance gene.

3.3.3.3 235 245 versus Westar – a Derived Bromoxynil Resistant Line versus Original Population Comparison

The differences obtained when 235 245, the derived bromoxynil resistant line, is compared to Westar, the original population, could be entirely due to the genetic difference between the 235 245 line and the Westar population and have nothing to do with the transgene or bromoxynil resistance. The performance of line 235 245 unsprayed was significantly different from the peformance of Westar only for oil content (0.6% lower) and protein content (0.6% higher) (Table 3.2). No biological cost appears to be associated with the bromoxynil resistance gene in this line. However, this is not the best comparison to detect biological cost since 235 245 is a single genotype selected at random from a population and could perform equal to, better than, or worse than the source population, Westar.

The 235 245 line sprayed was significantly different from Westar only for height (4 cm taller), days to flower (1 day earlier), and yield (7% lower) (Table 3.2). 235 245 (single line) and Westar 235 (isogenic population) contain the same bromoxynil resistance gene and yet no biological cost was associated with the resistance gene in Westar 235.

To date, there is only one published report on the effects of glufosinate ammonium (Liberty Link) herbicide resistance on the agronomic performance and seed quality traits of oilseed rape (Kumar *et al.* 1998). They evaluated the growth, development, and productivity of nineteen unsprayed glufosinate ammonium tolerant and susceptible isogenic pairs. Each tolerant line was obtained from a single transformation and contained either one or two inserts of the resistance gene. Kumar *et al.* (1998) observed delayed flowering and maturity, lower biomass and seed yield, and lower oil and protein content in thirteen of the tolerant lines. The five lines with two inserts of the resistance gene were more negatively affected than the lines with only one insert. Only six tolerant lines were similar in agronomic performance and seed quality to their susceptible isogenic pair. Therefore, for some transformations there appears to be a biological cost associated with the incorporating the glufosinate ammonium resistance gene in oilseed rape. These results do not agree with the present biological cost trial. The bromoxynil resistance gene did not affect days to flower and maturity, yield, or oil and protein content for both isogenic populations and the transgenic line. In some instances, positive effects were observed for yield.

There are no reports on the biological cost of glyphosate (Roundup Ready) or imazethapyr (Pursuit Smart) herbicide resistant varieties of oilseed rape.

The performance of isogenic bromoxynil resistant canola populations in these biological cost trials are very different from the results of triazine tolerant canola studies (Beversdorf *et al.* 1988; Beversdorf and Kott 1987; Holt *et al.* 1993; Grant and Beversdorf 1985; Holt and Thill 1994). The presence of triazine resistant cytoplasm resulted in a 20 to 30% yield reduction while this study observed no significant yield loss when bromoxynil resistance was incorporated into oilseed rape.

Seed quality for Westar 235, Westar 237, and 235 245 (unsprayed and sprayed) were typical for oilseed rape. The quality analysis indicated all the genotypes met the canola standards of less than 1% erucic acid in the oil and less than 30 µmole per gram glucosinolates in the air-dried oil free meal (data not shown) (Eskin *et al* 1996). These results were also observed by Arnoldo *et al.* (1992) and Kemble *et al.* (1991) when the neomycin phosphotransferase (NPTII) gene for kanamycin resistance was incorporated into *B. napus.* The oil and protein content profile for Westar 235, Westar 237, and 235 245 (unsprayed and sprayed) was also typical for oilseed rape. Beversdorf (1992) also observed a typical oilseed rape oil and protein content profile for glufosinate ammonium herbicide resistant oilseed rape.

3.3.4 Effect of Bromoxynil Resistance Gene

Since a bromoxynil herbicide spray effect could not be detected for the two transgenic populations and one transgenic line, in direct sprayed versus sprayed comparisons, an overall mean and least significant difference value was calculated for each genotype and compared to Westar to determine more accurately the effect of the bromoxynil resistance gene and derived herbicide resistance (Table 3.3).

When Westar 235 was compared to Westar over 17 station-years , Westar 235 was significantly different from Westar only for yield (8% higher) (Table 3.3). No significant differences were detected when Westar 237 was compared to Westar over 11 station-years. The transgenic line, 235 245, was significantly different from Westar only for days to flower (1 day earlier) and days to maturity (1 day earlier) when compared to Westar over 17 station-years.

There were few significant differences in these comparisons. In cases where a significant difference existed, bromoxynil resistance appears to have a positive effect on the performance of the transgenic bromoxynil resistant oilseed rape. Otherwise, bromoxynil resistance and the gene used to confer resistance appear to have no effect on the performance of the crop, i.e. no biological cost.

Table 3.3. Effect of bromoxynil resistance on lodging, plant height, days to flower, days to maturity, yield, oil content, protein content, and sum of oil and protein content means for two transgenic bromoxynil resistant populations and one transgenic bromoxynil resistant line compared to nontransgenic Westar population, 1994 to 1997

Genotype	Lodging (1-5)	Height (cm)	Days to Flower	Days to Maturity	Yield (kg/ha)	Oil (%)	Protein (%)	Sum[1] (%)	Station-Years
Westar	3.2	122	43	87	2258	45.6	25.0	70.6	17
W 235	3.0	125	43	87	2437*	45.5	25.2	70.7	17
LSD (0.05)	0.3	3.5	0.4	0.5	107	0.5	0.5	0.3	
Westar	3.2	129	43	87	2237	45.4	25.2	70.7	11
W 237	3.5	129	43	87	2341	45.2	25.4	70.7	11
LSD (0.05)	0.4	3.8	0.5	0.6	129	0.5	0.5	0.3	
Westar	3.2	122	43	87	2258	45.6	25.0	70.6	17
235 245	3.3	123	42*	86*	2332	45.2	25.4	70.6	17
LSD (0.05)	0.2	3.0	0.4	0.4	116	0.5	0.5	0.3	

*Indicates significance at the 5% level for within genotype comparison to Westar

[1]Sum=sum of oil and protein content

3.3.5 Summary

The purpose of this study was to compare the agronomic performance and seed quality traits of isogenic oilseed rape populations and a line to determine if a biological cost was associated with incorporating bromoxynil resistance gene into oilseed rape to degrade hydroxybenzonitrile herbicides such as bromoxynil. Since there does not appear to be consistent trends or consistent significant differences in the characteristics measured that can be attributed to bromoxynil resistance or the resistance gene itself, there is very little evidence of a biological cost. In fact, there are some indications of positive effects of the bromoxynil resistance gene and the bromoxynil resistance – a "biological benefit" instead of a biological cost.

4.0 OUTCROSSING STUDIES USING THE DOMINANT BROMOXYNIL RESISTANCE GENE IN OILSEED RAPE

4.1 INTRODUCTION

Oilseed rape is primarily a self-pollinating crop that possesses sticky, entomophilous pollen well suited to transfer by insects (Eisikowitch 1981). Researchers investigating outcrossing in oilseed rape, using elevated erucic acid levels as a marker, have reported a wide range in interplant outcrossing rates ranging from 5 to 75% (Rakow and Woods 1987; Lewis and Woods 1991). These results indicate oilseed rape readily outcrosses within its species, which can lead to contamination of breeding lines or unexpected genetic segregation in a field based plant breeding program. Prior to the incorporation of herbicide resistance into oilseed rape, phenotypic markers such as erucic acid levels and petal color were used to assess outcrossing rates. These phenotypic markers can be influenced by the environment and/or controlled by a number of genes, and are not as simple, apparent or definitive as a dominant herbicide resistance marker. The use of a phenotypic marker controlled by a single, nuclear dominant gene, such as bromoxynil herbicide resistance, provides an easy and accurate way to assess outcrossing rates in oilseed rape.

The purpose of this research was to determine the outcrossing rates between neighbouring oilseed rape plants, rows, and plots in typical plant breeding field

layouts using bromoxynil herbicide resistance as a marker. Outcrossing rates were investigated between neighboring 6-row plots, and also between neighboring single nursery rows at three different row spacings of 40, 80, and 120 cm. Additionally, the maximum plot to plant outcrossing rate was also determined.

4.2 MATERIALS AND METHODS

4.2.1 Outcrossing Rate Field Trials

Outcrossing rate field trials were established at the University of Manitoba Research Farm at Carman (hereafter "Carman"), at the Integrated Crop Management Systems Research Farm at Portage la Prairie (hereafter "Portage"), and at the University of Manitoba at Winnipeg (hereafter "Winnipeg") in 1996 and 1997.

Isogenic populations were used in three separate trial designs to determine the outcrossing rates in a typical *B. napus* plant breeding field layout. A University of Manitoba population designated as 03801 was used as the susceptible or "pollen recipient", while (03801 x 235-2-19, BC_4F_4) was used as the resistant line or "pollen donor". The isogenic populations differ only for the bromoxynil resistance gene, which is a single, nuclear, dominant gene.

A small plot belt cone seeder was used to seed the trials at a rate of 8 kg/ha. Carbofuran (10% granules) insecticide were banded with the seed at a rate of 12 kg a.i./ha to control flea beetles (*Phyllotreta cruciferae* Goeze and *P. striolata* F.). The Carman site was seeded on May 23, 1996 and May 15, 1997, the Portage site was seeded on May 30, 1996 and May 27, 1997, and the Winnipeg site was seeded on May 28, 1996 and May 26, 1997.

4.2.1.1 Plot to Plot Trials

This trial was designed so individual six-row susceptible (S) plots of summer rape (*B. napus*) were completely surrounded by eight, six-row bromoxynil resistant (R) plots (Figure 4.1). Eight bromoxynil susceptible plots were planted at each location. Individual plot size was 3 m long and 1.2 m wide and consisted of six rows of canola spaced 20 cm apart. The trials were seeded over eight ranges or blocks with 1.5 m wide pathway between ranges. Five bromoxynil resistant plots surrounded the two outside range susceptible plots.

Range	1	[R]	[R]	[R]	**[S]**	[R]
	2	[R]	**[S]**	[R]	[R]	[R]
	3	[R]	[R]	[R]	**[S]**	[R]
	4	[R]	**[S]**	[R]	[R]	[R]
	5	[R]	[R]	[R]	**[S]**	[R]
	6	[R]	**[S]**	[R]	[R]	[R]
	7	[R]	[R]	[R]	**[S]**	[R]
	8	[R]	**[S]**	[R]	[R]	[R]

Figure 4.1. Plot to Plot trial design grown at Winnipeg, Carman and Portage in 1996 and 1997. [R] = Bromoxynil resistant six-row plot, **[S]** = Bromoxynil susceptible six-row plot.

4.2.1.2 Row to Row Trials

This trial was designed so bromoxynil susceptible single rows were bordered by bromoxynil resistant rows (Figure 4.2). The nursery rows were planted using

three different row spacings (40, 80, 120 cm) to determine if row spacing influences outcrossing rate. The trials were seeded over eight ranges or blocks with 1.5 m wide pathway between ranges. Eight bromoxynil susceptible nursery rows, 3 m long, were planted for each row spacing at all locations. Due to field space constraints in 1997, the 120 cm row spacing was not planted at Carman.

The summer rape in the bromoxynil susceptible plots and susceptible rows were hand-cut at maturity, placed in jute bags, and subsequently threshed using a meticulously cleaned stationary thresher. Seed samples from the harvested plots were screened for bromoxynil resistance in the field the following summer.

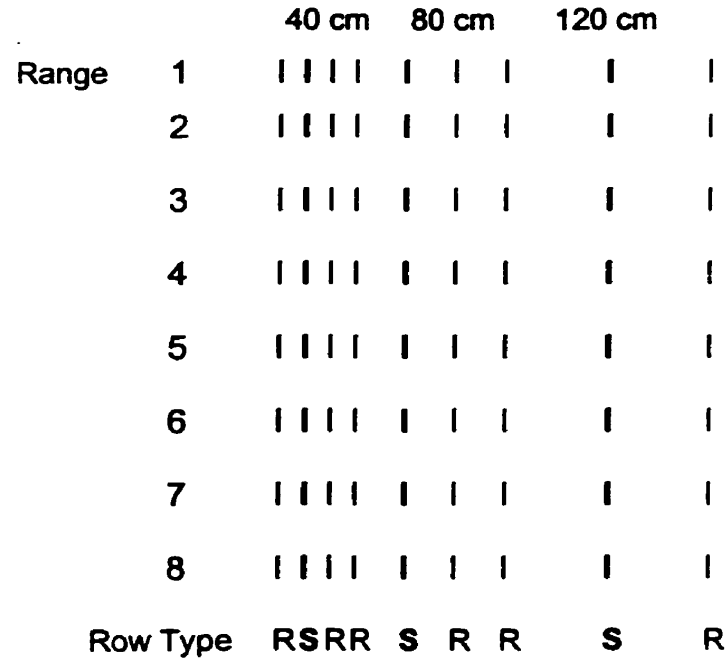

Figure 4.2. Nursery Row trial grown at Winnipeg, Carman, and Portage la Prairie in 1996 and 1997. R = Bromoxynil resistant row, **S** = Bromoxynil susceptible row. Not drawn to scale.

4.2.1.3 **Plot to Plant Trials**

This trial was arranged so one bromoxynil susceptible plant was grown in the center of a six-row bromoxynil resistant plot. In 1996, the trial was conducted only at the Winnipeg location. The small plot belt cone seeder was used to seed bromoxynil resistant plots. Individual plot size was 3 m long and 1.2 m wide consisting of six rows of bromoxynil resistant canola spaced 20 cm apart. The trial was seeded over eight ranges or blocks with 1.5 m wide pathway between ranges. Bromoxynil susceptible plants were transplanted between row 3 and 4 of the six-row plot at the two to four leaf stages.

The bromoxynil susceptible plants were seeded indoors on three different seeding dates in an attempt to synchronize plant development with the bromoxynil resistant plots in the field. Bromoxynil susceptible plants from two different seeding dates were transplanted in the field. For the first few weeks, these transplants were watered and fertilized approximately twice per week to encourage establishment and root development. Before the bromoxynil resistant plots bolted, the extra bromoxynil susceptible plants were removed and the bromoxynil susceptible plant closest to the bromoxynil resistant plot in development remained. An identification tag was placed on each bromoxynil susceptible plant so it could be easily identified. At maturity, the bromoxynil susceptible plant was removed and placed in a paper bag to dry. The bromoxynil susceptible plants were hand threshed and a sample of the seed was screened indoors for the presence of bromoxynil resistance. This design was replicated sixteen times at each site.

In 1997, the plot to plant trial was grown at Carman, Portage, and Winnipeg with the bromoxynil susceptible plants hand seeded immediately after the small plot cone seeder had seeded the bromoxynil resistant plots. The number of bromoxynil susceptible plants was thinned back so only one bromoxynil susceptible plant remained at flowering. This design was replicated sixteen times at each site.

4.2.2 Outdoor Screening Trials

Screening trials, to determine the outcrossing rate present in the outcrossing studies were conducted in the field at Winnipeg and Carman in 1997, and at Carman in 1998. Two screening trials were used each year. The screening trials were planted on May 30, 1997 at Winnipeg and June 18, 1997 at Carman. In 1998, the screenings trials were seeded on May 12, 1998 at Carman and May 27, 1998 at Carman. In both years, a small plot belt cone seeder was used to plant the trials at approximately 10 kg/ha. In 1997, the plots were 10 m long and 1.2 m wide at Winnipeg and 8 m long and 1.2 m wide at Carman. In 1998, the plots were 15 m long and 1.2 m wide in the first screening trial at Carman and 10 m long and 1.2 m wide in the second screening trial also at Carman. Plot size was selected in each case to optimize available land area while providing a reasonable number of seedlings in each plot.

The 1997 screening trials consisted of samples from the plot to plot trial and the row to row trial harvested in 1996. Four hundred and forty plots were planted in

an area approximately 0.6 ha. With an average emergence rate for seed planted of 42%, 224 667 seedlings were screened for the bromoxynil resistance trait in 1997.

The 1998 screening trial consisted of samples from the plot to plot trial and the row to row trial harvested in 1997. Four hundred and fifty-eight plots were planted in an area approximately 0.7 ha. With an average emergence rate for seed planted of 45%, 194 860 seedlings were screened for the bromoxynil resistance trait. The total number of seedlings screened for each treatment type in the 1997 and 1998 screening trials are presented in Table 4.1.

Emergence counts were conducted approximately 14 days after seeding on June 13m, 1997, July 2, 1997, May 29, 1998, and June 15, 1998. Plot stands in all screening trials were variable. Consequently, one representative row of the six-row plot was counted in every screening plot. The value obtained for the representative row was multiplied by six to give the plant stand of each screening trial plot.

The 1997 and 1998 screening trials were sprayed twice with bromoxynil herbicide at 750 g a.i./ha, the first spraying approximately 21 days after seeding and the second spraying approximately 5 days later. The first application took place on June 20, 1997, July 4, 1997, May 29, 1998, and June 17, 1998. A second application of bromoxynil was performed to confirm that the plants

Table 4.1. **Number of plots and seedlings screened in 1997 and 1998**

Year and Plot Type	Carman		Portage		Winnipeg	
	Plots (no.)	Seedlings (no.)	Plots (no.)	Seedlings (no.)	Plots (no.)	Seedlings (no.)
1997						
Plot to Plot	40	20 758	40	15 993	40	17 625
Row to Row						
40 cm	40	20 463	40	22 287	40	22 649
80 cm	40	22 264	40	18 077	40	24 281
120 cm	....	....	40	20 591	40	19 679
Total	120	63 485	160	76 948	160	84 234
1998						
Plot to Plot	42	29 296	42	26 323	42	16 294
Row to Row						
40 cm	34	9 431	31	9 927	30	10 289
80 cm	41	17 092	35	15 788	39	12 579
120 cm	42	15 082	42	20 182	38	12 577
Total	159	70 901	150	72 220	149	51 739
Combined over Years						
Total	279	134 386	310	149 168	309	135 973
Combined over Sites and Years						
Grand Total	898	419 527				

surviving the first spraying were truly resistant and not the result of a spray miss. The second spraying took place on June 24, 1997, July 8, 1997, June 4, 1998, and June 24, 1998. After the first spray, the number of bromoxynil resistant plants per plot were counted and recorded on June 22, 1997, July 6, 1997, June 1, 1998, and June 22, 1998. The plots were recounted after the second spray on June 26, 1997, July 10, 1997, June 5, 1998, and June 26, 1998. The number of resistant plants was then compared to the total number of seedlings per plot to calculate percent resistant individuals or outcrossing rate per plot.

4.2.3 Indoor Screening Trials

Screening trials to determine the outcrossing rate of the Plot to Plant trials were conducted in growth rooms at the University of Manitoba. One screening trial was conducted in the fall 1996 to screen the individual plants grown in Winnipeg in 1996, while two screening trials were necessary in the fall of 1997 to screen the individual plants grown in Winnipeg, Carman, and Portage in 1997.

The screening trials were planted on Oct 20, 1996, Oct 30, 1997, and Nov 3, 1997. A minimum of 150 seeds per plant were planted in 60 cell flats with metro mix. Two seeds per cell were grown to the one leaf stage and sprayed with bromoxynil at 560 g a.i./ha using a cabinet sprayer. The flats were sprayed approximately 10 to 14 days after planting on Oct 30, 1996, Nov 10, 1997, and Nov 14,1997.

The seedlings were assessed two days after spraying for resistance or susceptibility to bromoxynil. The number of bromoxynil resistant seedlings was compared to the total number of seedlings to determine the outcrossing rate. Approximately 9,970 seedlings were screened indoors for the presence of the bromoxynil trait (Table 4.2).

The outcrossing rate of a susceptible plant from the plot to plant trial was verified indoors to determine if it was truly the result of outcrossing. Twenty of the resistant seedlings from the outcrossed plant, after spraying with bromoxynil, were placed in a growth room and bagged at flowering to ensure selfing, and their progeny (F_2 families) screened for segregation with regards to the bromoxynil resistance trait. A segregating family would be the result of outcrossing and a non-segregating resistant family would be the progeny of a pure-breeding (homozygous) contaminant plant or part of a contaminant plant (such as a branch or pods).

4.2.4 Statistical Analysis

Data from the screening trials were subjected to analysis of variance techniques (SAS System for Windows v 6.21, SAS Institute Inc. Cary, North Carolina, USA). Since the plot to plant trial had 16 replicates and the plot to plot and row to row trials had eight replicates, the plot to plant trial was analyzed separately as a completely randomized design.

Table 4.2. Number of seedlings screened indoors in 1996 and 1997

Year and Plot Type	Carman Seedlings (no.)	Portage Seedlings (no.)	Winnipeg Seedlings (no.)
1996			
Plot to Plant	....	....	2 734
1997			
Plot to Plant	2 708	2 474	2 054
Combined over Years			
Total	2 708	2 474	4 788
Combined over Sites and Years			
Grand Total	9 970		

Although site grown was significant, a common occurrence in plant breeding/cultivar evaluation trials, it was of interest to compare treatment (also referred to as plot type) means using the entire data set. Error variances for the 40, 80, 120 cm row spacings and the plot to plot design were subjected to Bartlett's test for homogeneity of variances (Gomez and Gomez 1984). Since the error variances were heterogeneous and transforming the data set (square root and arc sine square root) did not correct this condition, a conservative procedure to test the significance of the main factors and interactions of the complete data set was followed as suggested by Cochran and Cox (1957). The procedure involved using the mean square error variance and associated degrees of freedom from the data subset with the highest error variance (40 cm row spacing) to test the significance of the main factors and interactions of the data set. This mean square error was used in calculating an LSD value to separate the treatments. This would result in a very conservative estimate of significant differences since this case is the most unfavorable that would occur. The complete data set was then analyzed as a split-split-plot over years and sites grown with replicate by year produced as the error term (Appendix Table 1). A paired t-test was performed to determine if the outcrossing rates for each plot type were significantly different from zero, at the 0.05 level.

Outcrossing rates have been measured in the outcrossing rate trials as the rate of contamination of bromoxynil susceptible plants, plots, and rows by pollen from

bromoxynil resistant plots and rows (i.e. unidirectional pollen mediated gene flow).

4.3 RESULTS AND DISCUSSION

4.3.1 Outcrossing Rate Field Trials

At five of the six sites, canola stands emerged uniformly and growth progressed normally. At Portage in 1997, emergence was variable and the subsequent plant stand was thin due to a hard, dry seedbed. This resulted in one of the 40 cm rows not being harvested. At all sites flowering synchrony between bromoxynil resistant and bromoxynil susceptible plots, rows, and plants was good, even at Portage in 1997.

4.3.2 Outdoor Screening Trials

There were minimal differences in counts of surviving canola seedlings after the first and second bromoxynil applications, indicating that bromoxynil spray coverage and herbicide activity was satisfactory and predictable. Furthermore, seedlings in the bromoxynil susceptible control plots were all killed with no seedlings escaping the herbicide application.

4.3.3 Outcrossing Rate Trials Combined over Sites and Years

Analysis of variance of the plot to plot and row to row outcrossing results indicated that site and plot type were highly significant (Appendix Table 1). Site and plot type represent approximately 25% and 38% of the treatment sums of squares for main factors, respectively.

4.3.3.1 Plot to Plot Trials

In all cases, the mean outcrossing rates were highly significantly different from zero (by t-test) (Table 4.3). Mean outcrossing ranged from 1.9% (±0.22) at Portage in 1996 to 6.2% (±0.27) at Winnipeg in 1996, with an overall mean of 4.0% (±0.23). Outcrossing for individual plots ranged from 1.3% at Portage in 996 to 7.7% at Winnipeg in 1996

In 1996, the plot to plot outcrossing rate was significantly higher at Winnipeg than at Carman while Carman was significantly higher than at Portage (Table 4.3). The difference in outcrossing rates over locations was probably due to pollinator activity, although systematic monitoring of insect pollinators was not conducted. There were honeybee colonies present near the outcrossing trials in Winnipeg and Carman, and insect pollinator activity was casually observed in the field during the growing season at these locations. In contrast, Portage appeared to have low insect pollinator activity. The reasons for the low insect pollinator activity are unknown although it appears that there were no bee colonies in the immediate vicinity of the Portage trial.

In 1997, differences in outcrossing rates between the sites were less pronounced. Winnipeg, with large number of bees in the immediate vicinity was significantly higher than Carman and numerically higher than Portage.

Table 4.3. Outcrossing Rate Summary, Plot to Plot Trials

Year & Site Grown	Mean (%)	S.E.	Range		Resistant Seedlings (no.)	Total Seedlings Screened (no.)
			Min %	Max %		
1996						
Carman	4.8b[1]	0.36	3.4	6.5	995	20 758
Portage	1.9c	0.22	1.3	3.2	304	15 993
Winnipeg	6.2a	0.27	5.4	7.7	1 095	17 625
1997						
Carman	3.5b	0.42	2.2	5.7	1 022	29 296
Portage	3.7ab	0.37	2.7	5.1	974	26 323
Winnipeg	4.7a	0.24	3.2	5.7	751	16 294
Combined over Years						
Carman	4.1b	0.32	2.2	6.5	2 017	50 054
Portage	3.0c	0.31	1.3	5.1	1 278	42 316
Winnipeg	5.4a	0.27	3.2	7.7	1 846	33 919
Combined over Sites						
1996	4.3a	0.41	1.3	7.7	2 354	54 374
1997	3.8a	0.21	2.2	5.7	2 747	71 913
Combined over Sites and Years						
	4.0	0.23	1.3	7.7	5 101	126 289

[1]Means (under each bold subheading) followed by the same letter were not significantly different at the 5% level.

Of the summer or winter rape outcrossing studies published to date, only one has investigated outcrossing between plots. Hühn and Rakow (1979) measured outcrossing in five low erucic cultivars of winter oilseed rape using five cultivars of winter oilseed rape with elevated erucic acid content as a marker. Eighteen plants were selected from the low erucic plots and 80 seeds from each plant were analyzed individually for erucic content using paper chromatography. They reported interplot outcrossing of 2.8% to 7.7% in northern Germany. These results agree with the results observed in the plot to plot outcrossing rate trial even though a relatively small sample (1,440 seeds per cultivar) was used by Hühn and Rakow (1979), in contrast to the 126,289 seedlings screened in the plot to plot trials in this study.

4.3.3.2 Row to Row Trials, 40 cm spacing

In all cases, the mean outcrossing rates were significantly different from zero (by t-test) (Table 4.4). Mean outcrossing ranged from 4.6% (±0.32) at Portage in 1996 to 13.1% (±1.82) at Winnipeg in 1997, with an overall mean of 9.5% (±0.62) (Table 4.4). Outcrossing rates for individual rows were variable, ranging from 3.2% at Portage in 1996 to 24.9% at Winnipeg in 1997, nearly an 8 fold difference in row to row outcrossing rates from site to site.

In both years (1996 and 1997), outcrossing rates observed at Carman and Winnipeg did not differ significantly. Similar to the plot to plot outcrossing rate results, in 1996 the outcrossing rate was significantly lower at Portage than at

Table 4.4. Outcrossing Rate Summary, Row to Row Trials, 40 cm spacing

Year & Site Grown	Mean (%)	S.E.	Range		Resistant Seedlings (no.)	Total Seedlings Screened (no.)
			Min %	Max %		
1996						
Carman	10.9a[1]	0.87	7.4	15.3	2 232	20 463
Portage	4.6b	0.32	3.2	5.7	1 026	22 287
Winnipeg	12.2a	1.23	7.9	19.4	2 775	22 649
1997						
Carman	9.5ab	0.76	7.3	12.8	901	9 431
Portage	7.5b	1.51	4.3	16.3	746	9 927
Winnipeg	13.1a	1.82	8.1	24.9	1 349	10 289
Combined over Years						
Carman	10.4a	0.58	7.3	15.3	3 133	29 894
Portage	5.5b	0.80	3.2	16.3	1 772	32 214
Winnipeg	12.5a	1.07	7.9	24.9	4 124	32 938
Combined over Sites						
1996	9.2a	0.84	3.2	19.4	6 033	65 399
1997	10.1a	0.86	4.3	24.9	2 996	29 647
Combined over Sites and Years						
	9.5	0.62	3.2	24.9	9 029	95 046

[1]Means (under each bold subheading) followed by the same letter were not significantly different at the 5% level.

Winnipeg or Carman (Table 4.4). Again in 1997, outcrossing rate means were lower at Portage than Winnipeg and Carman, but significantly lower only when compared to Winnipeg.

4.3.3.3 Row to Row Trials, 80 cm spacing

In all cases, the mean outcrossing rates were significantly different from zero (by t-test) (Table 4.5). Mean outcrossing rates ranged from 2.2% (±0.15) at Portage in 1996 to 8.2% (±1.00) at Winnipeg in 1997, with an overall mean of 5.6% (±0.37). Outcrossing rates for individual rows were variable, ranging from 1.5% at Portage in 1996 to 14.3% at Winnipeg in 1997.

In 1996, the row to row outcrossing rates at all three sites differed significantly showing similar trends to the plot to plot and the 40 cm row spacing trials. In 1996, the outcrossing rate was significantly higher at Winnipeg and Carman than at Portage (Table 4.5). In 1997, outcrossing rates were significantly higher at Winnipeg as compared to both Carman and Portage. The bee colonies maintained in the immediate vicinity are probably the cause of the elevated outcrossing rates in Winnipeg.

4.3.3.4 Row to Row Trials, 120 cm spacing

In all cases, the mean outcrossing rates were significantly different from zero (by t-test) (Table 4.6). Mean outcrossing ranged from 2.2% (±0.15) at Portage in 1996 to 6.5% (±0.52) at Winnipeg in 1996, with an overall mean of 3.9% (±0.25).

Table 4.5. Outcrossing Rate Summary, Row to Row Trials, 80 cm spacing

Year & Site Grown	Mean (%)	S.E.	Range Min %	Range Max %	Resistant Seedlings (no.)	Total Seedlings Screened (no.)
1996						
Carman	6.3b[1]	0.59	3.2	9.0	1 401	22 264
Portage	2.2c	0.15	1.5	2.7	395	18 077
Winnipeg	7.9a	0.51	5.4	10.2	1 929	24 281
1997						
Carman	4.0b	0.42	2.5	6.1	684	17 092
Portage	5.2b	0.50	3.8	7.6	821	15 788
Winnipeg	8.2a	1.00	5.3	14.3	1033	12 579
Combined over Years						
Carman	5.3b	0.46	2.5	9.0	2 085	39 356
Portage	3.6b	0.46	1.5	7.6	1 216	33 865
Winnipeg	8.0a	0.56	5.3	14.3	2 962	36 860
Combined over Sites						
1996	5.8a	0.53	1.5	10.2	3 725	64 622
1997	5.6a	0.53	2.5	14.3	2 538	45 459
Combined over Sites and Years						
	5.6	0.37	1.5	14.3	6 263	110 081

[1]Means (under each bold subheading) followed by the same letter were not significantly different at the 5% level.

Outcrossing rates of individual rows ranged from 1.6% at Portage in 1996 to 9.4% at Winnipeg in 1996.

In 1996, similar to the plot to plot, 40 cm row spacing, and 80 cm row spacing results, the outcrossing rate was significantly higher at Winnipeg than at Portage (Carman did not have the 120 cm row spacing in 1996 due to constraints on available land) (Table 4.6). In 1997, outcrossing rates were significantly different at all three sites, with Winnipeg having the highest rate and Carman the lowest. Similar to the 80 cm row spacing results in 1997, the outcrossing rate at Winnipeg was approximately twice that of Carman. Again, the bee colonies maintained in the immediate vicinity are probably the cause of the elevated outcrossing results at Winnipeg.

4.3.4 Comparison of Plot to Plot and Row to Row Outcrossing Rate Trials

Plot type had a significant effect on the outcrossing rates observed in the outcrossing trials (Table 4.7). Outcrossing rates observed in the row to row trial for the 40 cm spacing were significantly higher than the other plot types used in this study. Statistical differences were also detected between the 80 and 120 cm spacing of the row to row trial. These results indicate increasing the spacing between nursery rows decreases outcrossing, however it does not appear to be an effective method to virtually eliminate pollen mediated gene flow. Outcrossing rates for the plot to plot trial were significantly different from the 40 and 80 cm row spacings but not the 120 cm spacing.

Table 4.6. Outcrossing Rate Summary, Row to Row Trials, 120 cm spacing

Year & Site Grown	Mean (%)	S.E.	Range Min %	Range Max %	Resistant Seedlings (no.)	Total Seedlings Screened (no.)
1996						
Carman	...	...	...	...	...	...
Portage	2.2b[1]	0.15	1.6	3.0	453	20 591
Winnipeg	6.5a	0.52	4.8	9.4	1 281	19 679
1997						
Carman	2.4c	0.23	1.8	3.6	362	15 082
Portage	3.3b	0.29	2.3	4.7	660	20 182
Winnipeg	5.3a	0.24	4.3	6.4	667	12 577
Combined over Years						
Carman	2.4b	0.23	1.8	3.6	362	15 082
Portage	2.7b	0.21	1.6	4.7	1 113	40 773
Winnipeg	5.6a	0.31	4.3	9.4	1 948	32 256
Combined over Sites						
1996	4.7a	0.62	1.6	9.4	1 734	40 270
1997	3.7a	0.29	1.8	6.4	1 689	47 841
Combined over Sites and Years						
	3.9	0.25	1.6	9.4	3 423	88 111

[1]Means (under each bold subheading) followed by the same letter were not significantly different at the 5% level.

Table 4.7 Summary of Outcrossing Rate Trials, 1996 and 1997

Plot Type	Mean (%)	S.E.	Range Min %	Range Max %	Site-Years	Resistant Seedlings (no.)	Total Seedlings Screened (no.)
Plot to Plot	4.0c	0.23	1.3	7.7	6	5 101	126 289
Row to Row							
40 cm	9.5a	0.62	3.2	24.9	6	9 029	95 046
80 cm	5.6b	0.37	1.5	14.3	6	6 263	110 081
120 cm	3.9c	0.25	1.6	9.4	5	3 423	88 111
GRAND TOTAL						**23 816**	**419 527**

[1]Means followed by the same letter were not significantly different at the 5% level.

Outcrossing studies of oilseed rape published to date have not investigated outcrossing rates between nursery rows. Nursery or progeny rows are used in most *Brassica* breeding programs to evaluate breeding lines and make selections in early generations, and are typically spaced between 40 and 80 cm apart. Outcrossing rates observed in the row to row trials indicate that a considerable level of outcrossing occurs between nursery rows, particularly at the 40 cm row spacing. Increased levels of outcrossing at the 40 cm row spacing may be due to physical contact between oilseed rape plants. At 120 cm row spacing, physical plant contact was eliminated, therefore pollen transfer is due only to insects or wind.

The 6-row plots used in the plot to plot trial are commonly used by *Brassica* breeders to evaluate superior experimental lines in preliminary yield trials. The best lines are then advanced to replicated yield trials at two or more locations and the seed produced in the preliminary yield trials is used as the trial seed source. The results of the plot to plot trial indicate that a substantial amount of pollen is transferred between 6-row plots. A mean outcrossing rate of 4% in the plot to plot trial might be considered low, however, after a number of generations of 4% outcrossing, an unexpected, and possibly undesirable genetic shift in the breeding lines may occur.

The screening trials of the plot to plot and the row to row outcrossing trials identified 23,816 resistant individuals from 419,527 seedlings screened for the

presence of the bromoxynil resistance trait. Researchers should have confidence in the results of the plot to plot and row to row trials based on the large number of seedlings screened.

4.3.5 Plot to Plant Trials

In all cases, the mean outcrossing rates were significantly different from zero (by t-test) (Table 4.8). Mean outcrossing varied from 14.8% (±1.78) at Portage in 1997 to 26.4% (±3.84) at Winnipeg in 1996. The overall outcrossing rate mean value was 21.0% (±1.73). These trials were screened indoors. Germination for all outcrossed plants was greater than 95%, and seedling emergence was uniform. The bromoxynil susceptible controls were killed with no seedlings escaping the herbicide application.

As expected, outcrossing rates on an individual plant basis were quite variable, ranging from 5.8% at Portage in 1997 to 81.1% at Carman in 1997. The outcrossing rate of 81.1% was verified to determine if it truly was the result of outcrossing since the maximum reported outcrossing rate of a self-fertile oilseed rape line was 75% (Rakow and Woods 1987). The progeny of all 20 families derived from resistant putative F_1 plants, segregated with respect to the resistance trait and confirmed the 81.1% outcrossing rate for this particular plot to plant trial.

Table 4.8. Outcrossing Rate Summary, Plot to Plant Trials, Indoor Screening Results

Year & Site Grown	Mean (%)	S.E.	Range Min %	Range Max %	Plants Screened (no.)	Resistant Seedlings (no.)	Total Seedlings Screened (no.)
1996							
Winnipeg	26.4	3.84	8.2	55.0	14	721	2 734
1997							
Carman	19.5ab	4.34	7.1	81.1	16	525	2 708
Portage la Prairie	14.8b	1.78	5.8	27.5	15	366	2 474
Winnipeg	23.2a	2.58	12.1	40.0	14	477	2 054
Combined over Years							
Winnipeg	25.0	2.30	8.2	55.0	28	1 198	4 788
Combined over Sites							
1997	19.0	1.87	5.8	81.1	45	1 368	7 236
Combined over Sites and Years							
	21.0	1.73	5.8	81.1	59	2 089	9 970

[1]Means (under each bold subheading) followed by the same letter were not significantly different at the 5% level.

The majority of self-fertile oilseed rape outcrossing rate studies published involved interplant outcrossing, one pollen receptor surrounded by several pollen donor plants, similar to the design used in the plot to plant trial.

In Sweden, Olsson (1960) studied interplant outcrossing rates using petal color as the phenotypic marker. One white (recessive) flowered oilseed rape plant was completely surrounded by yellow (dominant) flowered oilseed rape plants. He reported a mean outcrossing rate of 34.6% in winter oilseed rape. Persson (1956 as cited by Lewis and Woods) completed a similar outcrossing rate study in winter and spring oilseed rape with petal color as the marker. He observed a mean outcrossing rate of 28.5% in winter rape and 36.0% in spring rape. The results of these studies reasonable agree with the mean outcrossing rate of 21.0% obtained in the current plot to plant study. The difference in outcrossing rates is probably due to the environment studied, sample size, and the marker used. The researchers did not indicate how many plants were screened or whether flowering was synchronous between the two cultivars and of these, both factors greatly influence outcrossing rates. Lewis and Woods (1991) indicated the results of the outcrossing studies that used petal color as a marker were biased because honeybees were sensitive to flower color. Petal color differences, therefore, do not provide researchers with an accurate measure of outcrossing rates in oilseed rape.

Becker *et al.* (1992) investigated outcrossing rates in oilseed rape using isozyme polymorphisms in five locations (3 sites in Sweden, 1 site in Denmark, and 1 site in northern Germany) to determine if location had an effect on outcrossing rate. At each location, 30 seeds from eight plants were analyzed for polymorphisms. Outcrossing rates, across the locations, ranged from 12 to 47% with a mean outcrossing rate of 34%. The lowest rate of outcrossing (12%) was observed in the most northern location (northern Germany), and the other sites ranged between 32 and 47%. Becker *et al.* (1992) demonstrated environment did affect outcrossing, which agrees with the plot to plant study since differences in mean percent outcrossing were detected between Carman, Portage la Prairie, and Winnipeg in 1997.

The outcrossing rates reported by Becker *et al.* (1992), however disagree with the outcrossing rates obtained in the present plot to plant trial. The differences are likely due to the sample size, and the marker used. In the plot to plant study, 9,970 seedlings from 59 plants were screened for the presence of the bromoxynil resistance trait while Becker *et al.* (1992) screened 240 seeds from 8 plants for the presence of isozyme polymorphisms. This extremely small sample size reduces the accuracy of the estimate and the ability to obtain the same result in subsequent experiments. Furthermore, the use of isozyme polymorphisms to measure outcrossing rate in oilseed rape requires expensive, specialized equipment and is very labor intensive. The use of isozyme polymorphisms is not

the most effective marker to provide an accurate estimate of outcrossing rate in oilseed rape.

In Scotland, Gowers (1981) used stem color differences, purple (dominant) and green (recessive), to measure interplant outcrossing rate in three cultivars of oilseed rape. Approximately, 250 seedlings of each cultivar were evaluated for the presence or absence of purple stem color. Gowers (1981) reported a mean outcrossing rate of 18% based on 750 seedlings screened which agrees with the mean percent outcrossing rate of 21% based on 9,970 seedlings screened observed in the plot to plant trials. However, range of outcrossing rates observed for individual plants between the two studies did not agree. Gowers (1981) reported individual plants ranged from 4% to 38% while the outcrossing rates of individual plants in the current plot to plant trials ranged from 5.8% to 81.1%.

The difference is possibly due to the environments studied. Gowers (1981) conducted his outcrossing at one site in Scotland during one growing season while the plot to plant trials were conducted at Winnipeg in 1996 and at Winnipeg, Carman, and Portage in 1997. The Carman and Winnipeg sites observed the highest rates of outcrossing but these sites had bee colonies in the immediate vicinity. Stem color provides an estimate of outcrossing rates in oilseed rape, however, the penetrance of stem color can be influenced by environment, therefore it is not as accurate or definitive a marker as bromoxynil herbicide resistance.

The outcrossing rates observed in the plot to plant trial confirm the outcrossing rates reported by Rakow and Woods (1987). Rakow and Woods (1987) used elevated erucic acid content to measure the outcrossing rate of oilseed rape in Saskatoon, Saskatchewan over a three year period. They measured the seed erucic acid content on individual seeds with gas liquid chromatography. In total they screened 3,400 seeds from 92 low erucic acid plants. The mean interplant outcrossing rate of 21.8 % reported by Rakow and Woods (1987) agrees with the mean interplant outcrossing rate of 21.0% based on the 9,970 seedlings screened in the present plot to plant trials.

The outcrossing rate of individual plants ranged from 2% to 75% in the study by Rakow and Woods (1987) and this agrees closely with the 5.8% to 81.1% range observed in the present plot to plant trial. The similarities between these two trials are likely due to the large sample size screened. It appears erucic acid content provides an accurate estimate of outcrossing in oilseed rape. However, erucic acid content is not as easy or as definitive to quantify as bromoxynil herbicide resistance.

Lewis and Woods (1991) also used erucic acid content to measure outcrossing in oilseed rape over a two year period in Alberta. Approximately 20 seeds from 278 plants were analyzed individually using paper chromatography to detect the presence or absence of erucic acid. They estimated the mean interplant

outcrossing rate was 50% which is much higher than seen in the plot to plant trials in this study (21%).

The difference in the outcrossing rates is likely due to the sample size screened, and the method used to detect the presence or absence of erucic acid. The plot to plant trial screened 9,970 individuals from 59 oilseed rape plants or approximately 169 seedlings per susceptible plant compared to 20 seeds per plant for 278 plants analyzed for erucic acid content by Lewis and Woods (1991). This small sample size reduces the accuracy of the outcrossing rate estimate. The paper chromatography method described by Lewis and Woods (1991) does not appear to be as accurate as the gas liquid chromatography method used by Rakow and Woods (1987) and could be the reason for the differences in reported interplant outcrossing rates.

The studies outlined above reported generally higher mean outcrossing rates in oilseed rape compared to the current plot to plant trial. However, these other studies used less definitive markers than dominant-trait herbicide resistance (e.g. petal color, isozyme polymorphisms, and erucic acid levels) and screened much, lower total numbers of seeds/plant and fewer plants than the 9,970 seedlings screened in the plot to plant trial conducted in this study.

4.3.6 Summary

The purpose of this study was to determine the outcrossing rates between neighbouring oilseed rape plants, rows, and plots within a typical plant breeding field layout using bromoxynil herbicide resistance as a marker. Outcrossing rate was measured in one direction, from resistant plots and rows of oilseed rape to susceptible plants, plots, and rows of oilseed rape. A significant outcrossing rate was detected and measured in all plot types. In total 419,527 seedlings were screened for the presence of the bromoxynil resistance gene from the plot to plot and row to row outcrossing rate trials conducted at three sites over a two year period. The 23,816 resistant individuals identified were the result of outcrossing events. The plot to plant or maximum outcrossing trial identified 2,089 resistant individuals from the 9,970 seedlings screened. These results indicate that pollen mediated gene flow does occur in typical summer rape plant breeding field layouts and that contamination of breeding lines will occur if pollination control measures are not utilized.

5.0 GENERAL SUMMARY AND CONCLUSIONS

Modern crop improvement/plant breeding includes the insertion of new gene(s) from unrelated organisms, using genetic engineering techniques, to resist herbicides. However, there is evidence that in certain instances the novel herbicide resistance could affect the phenology and/or performance of the crop under field conditions. The gene conferring bromoxynil herbicide resistance in *Brassica napus* was isolated from a soil bacterium and incorporated into oilseed rape. The biological cost of the transgene insertion and derived herbicide resistance was studied. When compared to Westar, few or no consistent differences were observed for the isogenic bromoxynil resistant populations, Westar 235 and Westar 237, and the derived bromoxynil resistant line, 235 245, in agronomic performance or seed quality characteristics. Importantly, there was no yield or seed quality penalty associated with the transgenics.

When Westar 235, Westar 237, and 235 245 were sprayed with bromoxynil and compared to their respective genotypes unsprayed, there were no significant differences in agronomic performance and seed quality characteristics with a single exception for yield of line 235 245. Since both constructs were tested in isogenic populations and line, the data suggests that there is no biological cost associated with bromoxynil resistance or inserting the resistance gene in oilseed rape. In some instances, a positive effect or "biological benefit" is associated with bromoxynil resistance and the bromoxynil resistance gene. Given these

results, plant breeders can continue to develop new cultivars of bromoxynil resistant oilseed rape without concern about a biological cost associated with the resistance gene or the derived herbicide resistance.

The outcrossing rates of summer rape observed in this study were significantly different from zero indicating a substantial amount of pollen mediated gene flow does occur in a typical oilseed rape plant breeding field situation in western Canada. Plot type had a significant effect on the outcrossing rates observed at all three sites in both years. The highest mean outcrossing rate (9.5%) was obtained for the 40 cm row spacing of the row to row trial where pollen transfer occurred through physical contact. Mean outcrossing rates decreased significantly as row spacing increased. However, the mean outcrossing rate for the 120 cm row spacing was still high (3.9%) considering pollen transfer is mediated only by insects and wind at this row spacing. The mean outcrossing rate for the 6 row plots was 4.0%. These outcrossing rates demonstrate oilseed rape readily outcrosses within its species and breeding lines can be contaminated in an oilseed rape plant breeding program unless pollination control methods are employed.

In the field, the use of tents is the best method to eliminate pollen mediated gene flow between genetically different lines of oilseed rape and maintain the genetic purity of breeding lines. Individual plants can also be bagged to ensure self-pollination. However, this pollination control method is labor intensive and the

bagged plants usually suffer from disease and insect problems. In the absence of pollination control measures, nursery or progeny rows should be spaced at least 80 cm apart and genetically similar breeding lines grouped together in the field. This will help reduce genetic drift for important agronomic and seed quality characteristics, but increase the amount of land required to evaluate breeding lines. An additional way to maintain genetic purity is to use remnant seed from crosses in the greenhouse for each field season. Genetic purity would be maintained, however a generation of genetic advance is lost. Doubled-haploid techniques can be used to produce breeding lines completely homozygous at all loci and eliminate up to 6 generations in a conventional breeding program. Remnant seed of doubled-haploid lines would maintain genetic purity and a generation of genetic advance would not be lost. This study indicates that the genetic purity of breeding lines harvested at the end of the growing season could be 10% different from the seed planted in the spring if a pollination control measure is not used (based on the outcrossing rates observed in this study for the 40 cm row spacing). Within a few generations a considerable genetic shift could occur.

It is important to note that in this study the outcrossing rate of oilseed rape has been measured as the rate of contamination of bromoxynil susceptible plants, plots, and rows by pollen from bromoxynil resistant plots and rows. Most other studies published in the literature have also measured only unidirectional pollen mediated gene flow. Total outcrossing involves pollen mediated gene flow in two

directions. The use of two different, definitive, easy-to-use markers like herbicide resistance (e.g. bromoxynil and glyphosate) would allow breeders to accurately assess the total outcrossing rate of oilseed rape. As a first approximation, doubling the unidirectional outcrossing rate obtained in this study will give an estimate of the total outcrossing rate observed for summer rape in western Canada.

6.0 LITERATURE CITED

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Appendix Table 1. General Linear Models (GLM) analysis for row to row and plot to plot outcrossing rate trials combined over plot types

Factor	Df	Sum of Squares Type III	F-(statistic)
Replicate	7	54.7764	0.73 NS
Site	2	533.8990	24.84 *
Plot Type	3	824.1482	21.57 *
Year produced	1	0.0546	0.01 NS
Repicate*Year produced (error a)	7	34.4748	
Replicate*Site	14	40.6742	0.270 NS
Replicate*Plot Type	21	81.5270	0.361 NS
Site*Year produced	2	108.4060	5.044 *
Site*Plot Type	6	79.2000	1.228 NS
Year produced*Plot Type	3	21.5680	0.669 NS
Error b	35	376.0781	

NS=non-significant

*=significant at 5% the level

Appendix Table 2. General Linear Models (GLM) analysis of completely randomized design for the plot to plant outcrossing trials combined over sites and years

Factor	Df	Sum of Squares Type III	F-(statistic)
Site	2	1042.3248	3.13 NS
Year produced	1	61.8057	0.37 NS
Error	55	9165.2898	

NS=non-significant