AN ABSTRACT OF THE DISSERTATION OF

Michael P. Quinn for the degree of Doctor of Philosophy in Crop Science presented on October 4, 2010.

Title: Potential Impacts of Canola (*Brassica napus* L.) on *Brassica* Vegetable Seed Production in the Willamette Valley of Oregon

Abstract approved:

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Carol A. Mallory-Smith

In the Willamette Valley of Oregon, a combination of the need for rotational crops and an increased desire for biofuel production created interest in planting *Brassica napus* (canola). However, questions were raised arisen over the potential damage canola production could have on the preexisting *Brassica* vegetable seed industry. To address these concerns three studies were conducted to: 1.) Determine the potential of gene flow and hybridization via pollen from *Brassica napus* to related *Brassica* vegetable crops; 2.) Evaluate whether transgenes will be detectable in harvested *Brassica* vegetable seed; 3.) Evaluate the potential for volunteer canola to become a contaminant in the *Brassica* vegetable seed crops. Crossing experiments were conducted in 2007, 2008, and 2009 using *Brassica rapa* or *Brassica oleracea* inbred line receptor plants placed within conventional *B. napus* fields. Once seed set occurred on the receptor plants, each was harvested individually and the seed germinated in a growth chamber. Flow cytometry, morphological and molecular

analyses were performed on the seedlings. Hybridization between *B. napus* and *B. rapa* inbreds was 74% in 2007, 89% in 2008, and 15% in 2009. However, no hybridization occurred between *B. napus* and the *B. oleracea* inbred lines. Experiments were conducted using transgenic *B. napus* and the previously mentioned vegetable species, to quantify outcrossing rates in a greenhouse environment. Transgenes were detectable in both germinable and non-germinable seed produced on non-transgenic plants. Following *B. napus* harvest at the field sites, shattered canola seed was collected from both windrow and non-windrow locations. Approximately 30 days after the shatter samples were taken, canola seedling recruitment counts were made in quadrats placed immediately adjacent to the location of the seed shatter samples. Results of this volunteer assessment indicated differences in seed shatter between fields and windrow vs. non-windrow locations, but seedling recruitment only differed by fields. These studies indicate that canola, if grown in the Willamette Valley, has the potential to hybridize with related *Brassica* vegetable species grown for seed. However, when managed properly, canola volunteer persistence is unlikely to be an issue within fields in the monocot crop rotations used in the Willamette Valley.

Potential Impacts of Canola (*Brassica napus* L.) on *Brassica* Vegetable Seed
Production in the Willamette Valley of Oregon

by
Michael P. Quinn

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APPROVED:

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Head of the Department of Crop and Soil Science

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Michael P. Quinn, Author

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COUNTRIBUTION OF AUTHORS

Dr. Carol A. Mallory Smith advised all aspects of the research conducted, as well as provided feedback throughout the project. Additionally, she was actively involved in the preparation and improvement of the manuscripts. Dr. James R. Myers provided both advice and plant material for the greenhouse and field crosses and assistance with the manuscript. Dr. Andrew Hulting also was involved with the improvement and preparation of the manuscript.

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CHAPTER 1: GENERAL INTRODUCTION

Oil seed rape or canola (*Brassica napus* L.) is an allotetraploid (*2n=4x=38*, AACC) originating from an ancient hybridization between the diploids *B. rapa* (*2n=2x=20*, AA) and *B. oleracea* (*2n=2x=18*, CC) (Ford et al. 2006). This relationship was first elucidated by U (1935), who documented that *B. napus* has genomes in common with *B. oleracea* and *B. rapa*. This close species relationship between diploid and allotetraploid *Brassica* species contributes to the ease with which interspecies crossing can occur (Meyers 2006).

Brassica napus appeared as a cultivated crop in Europe sometime in the early 1300's (Tsunda 1980). Most likely it originated at multiple locations along the northern Mediterranean and western European coast where the habitats of *B. rapa* and *B. oleracea*, feral or cultivated, overlapped. Olsson (1960) suggested that *B. napus* probably arose independently several times by spontaneous hybridization of different forms of *B. rapa* and *B. oleracea* growing in medieval gardens.

The taxonomy of the *Brassica* is still not resolved completely (Rubatzky and Yamaguchi 1999). A unique aspect of many of the *Brassica* crop species is that several different crops with varying morphologies are derived from the same species. Cabbage, kohlrabi, cauliflower, broccoli, Brussels sprouts, collards and kale are

derived from *B. oleracea*, while Chinese cabbage (pak choi and pe tsai), mizuna, broccoli raab, and turnip are all *B. rapa* (Rubatzky and Yamaguchi 1999).

Within the *Brassica* species varying levels of interfertility exists between species (Rieger et al. 2001) and reports vary greatly as to the extent of hybridization that can occur between species (Hancock 2004). A distinctive feature of *Brassica* species origin and evolution is the formation of allotetraploid species from hybridization of diploid progenitors (Olsson 1960). In general, viable crosses between diploids and allotetraploids occur more readily when the diploid parent has a genome in common with the allotetraploid parent. Whether these hybrids are viable, whether they will have restored fertility in subsequent generations and whether introgression of genes occurs in subsequent generations remain as important research questions (Chevre et al. 1998; Chevre et al. 2000; Jorgenson et al. 1996). Additionally, hybridization rates can vary depending on environment (Becker et al. 1992) and distance between the plants (Hall et al. 2000; Mesquida and Renard 1982; Timmons et al. 1995). Pollen flow from canola can travel long distances. Studies in Canada have found evidence of canola pollen movement up to 3 km (Rieger et al. 2002).

Sub-species of *B. rapa* vary in their level of cross-compatibility. Crosses are common between *B. rapa* and *B. napus*, though reported levels of hybridization vary (Brown, et al. 1995; Warwick, et al. 2003; Wilkinson, et al. 2000). Hybrids have been reported to have reduced fertility and lower seed production compared to the parents (Jorgensen and Andersen 1994). *Brassica oleracea* and *B. napus* hybridization is not common (Scheffler and Dale 1994) and hybrid progeny are difficult to obtain artificially (Chiang et al. 1997). However, hybrid progeny of a *B. napus* and feral *B.*

oleracea have been found in the wild (Ford et al. 2006), but at very low frequencies. While these hybridization events may be rare, the potential does exist for them to occur under field conditions.

Western Oregon, with its mild winters and dry summers, has the ideal climate for seed production. The Willamette Valley, in particular, has a specialty seed crop industry that produces both vegetable and flower seeds. While the *Brassica* specialty seed crop growing area is small, it can be very profitable, often netting a grower more than $4,000 per hectare depending on the seed crop grown (Ehrensing 2007). In fact, western Washington and western Oregon combined produce nearly all the world supply (~90%) of European cabbage, Brussels sprouts, rutabaga and turnip seed, and a substantial portion (20 – 30 %) of radish, Chinese cabbage and other Asian *Brassica* vegetable crops (Myers 2007). This production constitutes a significant portion of the global *Brassica* vegetable seed market; particularly European and Asian markets, as 50 to 60% of the seed grown is exported to these regions.

The combination of the need for broadleaf rotational crops within the grasses grown for seed cropping systems, and an increased desire for local biofuel production has created interest among growers to plant canola in the Willamette Valley. However, the specialty seed crop growers of western Oregon and Washington voiced concern about the potential negative impact growing canola in the region could have on the industry (Myers 2006). Very few other regions of the world have the unique climate to produce high quality *Brassica* vegetable seed. The *Brassica* vegetable seed crop production could be jeopardized if contamination occurs from canola hybridizing with vegetable varieties. The risk would be even greater if the crops were contaminated

with transgenic canola. International purchasers of the vegetable seed crops have extremely low tolerances for any contamination, and some maintain a zero tolerance for transgenic contamination (Tichinin 2007).

Hybridization studies among the species related to *B. napus* have primarily focused on gene flow to either *B. rapa* or to weedy relatives (Bing et al. 1996; Brown and Brown 1996; Jorgensen and Anderson 1994; Jorgensen et al. 1996; Lefol et al. 1995; Lefol et al. 1996; Warwick et al. 2003; Williams et al. 1986). Additionally, hybridization studies have not included gene flow to the *Brassica* vegetable crops (Myers 2006). Frequently if these crops are mentioned in published studies, the authors state that the vegetable crops are harvested before they flower so gene flow is not a concern. This conclusion is true if the crops are harvested prior to flowering, such as for fresh market crops, but not if they are being grown for seed production. A compounding factor that may increase outcrossing of *B. napus* to *Brassica* vegetable crops with is that many are male sterile or self-incompatible.

Therefore to address these issues, we addressed three general objectives: 1.) Determine the potential gene flow via pollen from *Brassica napus* to related *Brassica* vegetable crops; 2.) Evaluate whether transgenes will be detectable in harvested *Brassica* vegetable seed; 3.) Evaluate the potential for volunteer canola to become a contaminant in the *Brassica* vegetable seed crops.

CHAPTER 2: ASSESSMENT OF OUTCROSSING BETWEEN CANOLA (*Brassica napus* L.) AND RELATED BRASSICA VEGETABLE SPECIES

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ABSTRACT

In Oregon's Willamette Valley, a combination of need for broadleaf rotational

crops and an increased desire for local biofuel production has created interest among

growers for planting *Brassica napus* (canola). However, questions have arisen over the

potential damage large scale canola production could have on the existing *Brassica*

vegetable seed production industry. The reputation of the *Brassica* vegetable seed

production industry is based on the purity and the high quality of seed. In fact, a seed

lot may be rejected if more than three outcrossed seed per 1,000 seed is found. The

risk is even greater if the crops are cross pollinated with transgenic canola because

some international purchasers of the vegetable seed crops have zero tolerance for

transgenic contamination. While there is a great deal of information on hybridization

between canola and weedy species, very few studies address hybridization between

canola and related vegetable species. To address this issue, experiments were

conducted in 2007, 2008, and 2009 using *Brassica rapa* and *Brassica oleracea* inbred

lines as pollen receptors placed within a conventional (non GMO) *B. napus* field. Flow

cytometry, morphological analysis, and molecular markers were used to identify

hybridization between the species. Greenhouse crosses were conducted using either a

conventionally produced imazamox resistant or a transgenic glyphosate resistant *B.*

napus line as the pollen parent and either a self incompatible *B. rapa* var. *chinensis*

(Chinese cabbage) or cytoplasmic male sterile (CMS) *B. oleracea* var. *italica*

(broccoli) inbred lines as the maternal parent. Herbicide resistant *B. napus* lines were

used because they provide a reliable selectable marker for positive identification of a

cross. Results of the field experiments indicated that hybridization occurred 74% in

2007, 89% in 2008, and 15% in 2009 between *B. napus* and *B. rapa* inbred lines. However, no hybridization occurred between *B. napus* and either *B. oleracea* inbred line. Results of the greenhouse crossing experiments using *B. rapa* as the maternal parent resulted in hybridization rates which ranged from 0 to 15.3% depending on *B. rapa* var. *chinensis* inbred line, and on which herbicide resistant *B. napus* paternal parent was used in the cross. Greenhouse crosses using *B. oleracea* inbreds as the maternal parent produced no germinable seed, and none of the aborted seed tested positive for the presence of the transgene. Presence of transgenic material was detected in both germinable and non-germinable seed produced on non-transgenic *B. rapa* female plants in the greenhouse crosses. We believe this is the first documentation of transgenic material identification in non-germinable seed produced on non-transgenic plants. This research demonstrates that the potential exists for hybridization between canola and some *Brassica* vegetable species under field conditions.

Nomenclature: canola, *Brassica napus* L., *Brassica rapa*, *Brassica oleracea*

Key Words: vegetable seed, off types, outcrossing.

INTRODUCTION

As the demand for biofuels grows in the United States, there is increasing interest in producing oilseed crops. Frequently, moving production to new regions can influence the established agricultural practices in unanticipated ways. Factors such as cross contamination, transgenic or otherwise, via gene flow raise concerns for the established commodity growers. Additionally, the new crops may increase insect and disease pressure in regions where these pests were previously low. These issues can result in conflicts between producers due to either real or perceived threats to the existing industry (Goodman 2000).

Western Oregon has an ideal climate for the production of many seed crops. In the Willamette Valley of Oregon, 200,000 to 300,000 hectares of grasses grown for seed are harvested annually, and the specialty seed crop industry produces over 5,600 hectares of vegetable and flower seeds. In total, about half of the arable land in the Willamette Valley is devoted to seed production. Such a concentration of seed production can not be found anywhere else in the world (Tichinin 2007). The combination of the need for broadleaf rotational crop options with grass seed crops, and an increased desire for local biofuel production has created interest in growing *Brassica napus* (canola) for oilseed production. However, the specialty seed crop growers of Western Oregon and Washington voiced concern about potential negative impacts that growing canola in the region could have on their industry. Very few other regions of the world have the climate to produce high quality *Brassica* vegetable seed. Twenty-five hundred to 3,000 hectares of *Brassica* seed are grown in the Willamette

Valley annually, with a value of $10 to $12 million dollars (Ehrensing 2007). Fifty to 60% of the seed is exported to Europe and Asia, constituting a significant portion of the global *Brassica* vegetable seed market.

Hybridization studies among the species related to *B. napus* have concentrated on gene flow to either non-vegetable *B. rapa* sub-species or to weedy relatives (Bing et al. 1996; Brown and Brown 1996; Jorgensen and Anderson 1994; Jorgensen et al. 1996; Lefol et al. 1995; Lefol et al. 1996; Warwick et al. 2003; Williams et al. 1986). While these studies are of ecological and agricultural value, they do not address outcrossing of canola with *Brassica* vegetable species (Myers 2006). If related vegetable crops are mentioned, the authors state that are harvested before they flower so gene flow is not a concern. This is true if the crops are harvested prior to flowering, as fresh market crops, but not when being grown for seed. *Brassica* vegetable species grown for seed can be either fall planted as seed or spring planted as transplants. Depending on the method of planting, synchronization of flowering can occur with either fall or spring planted canola. Additionally, many of the vegetable crops are male sterile or self-incompatible so greater crossing would be expected to occur. In field experiments examining outcrossing in canola, hybridization rates vary depending on environment and distance between the plants (Beckie and Hall 2008).

Hybridization between *Brassica* species varies greatly (Chiang et al. 1977; Becker et al. 1992; Chevre et al. 2000). For example, *Brassica napus* is self-fertile, but outcrossing rates as high as 47% have been reported (Williams et al. 1986). Pollen flow between canola cultivars has been well documented. In Canada gene flow between transgenic lines was reported at 800 m, which was the limit of the study

(Beckie and Hall 2008). Canola volunteer plants have been identified containing transgenes for both Roundup Ready™ and Liberty Link™ traits resulting from natural pollen movement under field conditions (Scheffler and Dale 1994; Hall et al. 2000; Schafer et al. 2010). Outcrossing between adjacent canola fields with differing herbicide resistance traits has resulted in volunteer canola with both conventional and transgenic herbicide resistance (Beckie et al. 2003).

Pollen of *Brassica* species can be disseminated by insect pollinators and by wind (Mesquida and Renard 1992; Beckie et al. 2003). Typically insect pollinators, such as honey bees, are capable of moving pollen less than a few kilometers (Pasquet et al. 2008). Wind dispersed pollen moves much greater distances, in some cases tens of kilometers. Dual-vector outcrossing may explain the disparity in reported pollen dispersal from a few meters to 25 km that has been reported for canola (Timmons et al. 1995), making it difficult to predict the furthest distance that viable pollen can move. Therefore, it may not be possible to establish adequate buffer zones to prevent cross pollination of compatible *Brassica* species in the field.

The taxonomy and genetics of the *Brassica* species are complex. One of the unique aspects of the crop species is that several crops, exhibiting very different morphologies, were derived from the same species and are, therefore, highly interfertile (U 1935; Hancock 2004). Cabbage, kohlrabi, cauliflower, broccoli, Brussels sprouts, and kale originated from *B. oleracea,* while Chinese cabbage (pak choi and pe tsai), mizuna, broccoli raab, and turnip are *B. rapa* (Rubatzky and Yamaguchi 1999).

Species of *B. rapa* vary in their level of cross-compatibility (Olsson 1960).

Crosses are common between *B. rapa* and *B. napus* though reported levels of

hybridization vary widely (Brown, et al. 1996; Warwick, et al. 2003). Additionally, *B.

rapa* x *B. napus* hybrids have been found to have reduced fertility and lower seed set

compared to either parental species (Jorgensen and Andersen 1994). Hybridization of

B. oleracea and *B. napus* is rare. However, hybrid progeny of this cross have been

found in the wild (Ford et al. 2006). While these hybridization events may be rare, the

potential does exist for them to occur under field conditions.

Quantification of the impact canola may have on related *Brassica* vegetable seed

crops via outcrossing is of importance to the seed production sectors in the Willamette

Valley. The objectives of this study were: to determine the potential for gene flow and

hybridization via pollen flow from *B. napus* to related *Brassica* vegetable crops under

both greenhouse and field conditions, and evaluate whether transgenes could be

detected in the resulting viable and aborted seed.

MATERIALS AND METHODS

Field Experiments. Studies were conducted in 2007, 2008, and 2009 near Corvallis,

OR. In each year, one field was planted with conventional *B. napus* 'Athena' at the

commercial sowing rate (~ 9 kg/ha). Brassica vegetable seed inbred lines were

obtained from local sources. Accession numbers and inbred parental information are

propriety information for these lines; therefore, codes were used identify the inbreds

used in each cross. In 2007 a self-incompatible *B. rapa* var. chinensis (Pak choi)

inbred line (BRCF) and a cytoplasmic male sterile (CMS) *B. oleracea* var. *italica*

(broccoli) inbred (BOI) were grown in the greenhouse and moved into the field when

the *B. napus* began flowering, and returned to greenhouse after pollination. The source

of the CMS in the *B. oleracea* inbred lines was the 'Anand' cytoplasm (Cardi and

Earle 1997). In 2008 and 2009, *B. oleracea* var. *capitata* (BOCF, and BOCM) and a

B. rapa var. *pekinensis* (Pei tsai) inbred lines (BRPF) were used as receptor species in

the field experiments. These plants were grown in the greenhouse and moved to the

field during *B. napus* flowering. The greenhouse plants were planted sequentially to

ensure synchronization of flowering with the *B. napus*. Each *B. napus* x inbred line

field experiment was conducted independently to prevent cross pollination between

the receptor species. Isolation was achieved by placing only one receptor species in a

field at a time. Initiation and duration of flowering were recorded for each species.

Receptor plants were arranged in a 4 x 4 m grid inside the perimeter of a 15 x 15 m

study area with one plant located at the intersection of the grid axes. Seed of each

receptor plant were harvested individually. The seed were placed into 10.2 x 10.2-cm

germination boxes containing moistened blotter paper[1] and put into a germination

chamber set to a 24/17 C day/night temperature regime with a 13 h photoperiod

(Warman 1999). The number of germinated seedlings was recorded and germination

percentages calculated for each cross. Shrunken seed produced on the *B. oleracea* var.

italica (BOI) plants were tested for viability with a tetrazolium assay according to

methods described by the Association of Official Seed analysts (AOSA 2002).

Following germination counts, seedlings were removed from the growth chamber, transplanted into commercial potting soil[2], and transferred to the greenhouse with a 20/20 C day/ night temperature and no supplemental lighting. Once the seedlings reached the five leaf stage, approximately 1 cm^2 of leaf tissue was taken from each plant, and immediately placed on ice. Tissue samples were macerated in 2 ml LB01 buffer, incubated on ice for 5 min, then filtered through a 50 μm screen. The extract was placed in a centrifuge and spun for 10 min at 1000 rpm until the DNA pelletized. The supernate was removed and the pellet was resuspended in 300 μl of a 25 μg/ ml propidium iodide solution for 10 min. Ploidy level of the plants was determined by flow cytometry with a Beckman Coulter FC 5000[3] using a forward log (FL2) scale at 639 volts. Data analysis was conducted with the Beckman Coulter CXP software package[4] using the parental inbred lines (*B. rapa* or *B. oleracea*) and *B. napus* as relative positive controls in each sample run. *B. napus* is an allotetraploid (*2n=4x=38, AACC*), while both lines of the *B. rapa* (*2n=2x=20, AA*) and *B. oleracea* (*2n=2x=18, CC*) are diploids. Therefore, hybrids of *B. napus* and the receptor species are triploid and readily distinguishable using this technique.

In addition to sampling leaf tissue from each of the seedlings, a morphological assessment was used to determine potential hybrids. Morphological descriptors such as color, shape and size of vegetative and reproductive structures have been widely used in taxonomic studies of the *Brassica* species (Gomez-Campo 1980). Seedlings were visually evaluated and rated as either the result of a self fertilization, or hybridization of the respective receptor species and *B. napus* based upon morphological characteristics defined by Musil (1950). For the progeny of each cross,

leaf shape, color, presence/absence of hair on leaves and stems, and stem shape was noted. These characteristics were then compared to those of the maternal inbred and those of *B. napus*. In the case of the *B. rapa* inbred maternal plants, self fertilizations produced progeny displaying a light green leaf color, an obelliptic shaped leaf with a prominent midrib, and no hair on either stems or leaves. These offspring appeared identical to the maternal parent. However, putative hybrid individuals produced on the *B. rapa* inbred maternal plants had a darker blue green leaf color, incised leaf with reduced midrib, and pubescence on both leaves and stems. These individuals exhibited morphological characteristics of *B. napus*. In the case of the *B. oleracea* inbred maternal plants, self fertilizations produced progeny with a dark green leaf color, a thick ovate shaped leaf, and glabrous stems and leaves. These offspring appeared identical to the maternal parent. This evaluation was conducted before cytological analysis to avoid introducing bias into the results. The results of both the flow cytometry and molecular analyses were then compared to examine the accuracy of the morphological assessment.

Hybrids between *B. napus* and either of the receptor species can be detected using molecular marker analysis. Primer pairs corresponding to the A genome of *B. napus* (Iniguez-Luy et al. 2006) were used to identify hybrid individuals in the progeny of the field crosses. We selected Primer pair 7, which amplifies the A genome from *B. napus* but not from *B. rapa* var. *chinensis* (BRCF) or *B. rapa* var. *pekinensis* (BRPF) receptor species. This marker was effective in screening for hybrids between the *B. oleracea* varieties and *B. napus* because *B. oleracea* does not have an A genome. Therefore, any progeny in which Primer 7 amplified, would be a hybrid. For

this analysis total genomic DNA was extracted from young leaves using the DNeasy

96 Plant kit[5] (Qiagen). The PCR reaction mixture (10 µL) contained 2-5 ng of

genomic DNA, 0.2 µL each DNTP, 0.2 µL each primer, 1 µL 10X buffer, and 0.06 µL

Taq DNA Polymerase[6] (Qiagen). The PCR program consisted of: 1 min at 95 C,

followed by 35 cycles of 30 s at 95 C, 30 s at 60 C (primer specific), and 45 s at 72 C,

with a final extension of 10 min at 72 C, using a C1000™ Thermal Cycler[7] (Bio-Rad).

Uniformity of PCR amplification was resolved by UV fluorescence after

electrophoresis on 2% agarose gel with ethidium bromide. The results of the marker

screening were compared to both the cytological and morphological assays to check

for discrepancies among the screening methods.

Greenhouse Experiments. *Brassica* vegetable seed inbred lines were obtained from

local sources. Accession numbers and inbred parental information are propriety

information for these lines, therefore codes were used identify the inbreds used in each

cross. Isolated greenhouse crossing experiments were conducted using either

Clearwater®, an imazamox resistant (IMI) or DKL38-25 a glyphosate resistant (RR) *B.*

napus (canola) cultivar as the pollen parents, and two self-incompatible *B. rapa* var.

chinensis (Pak choi) vegetable seed inbred lines (BRCM and BRCF) as receptor

plants. Crosses were conducted with an inbred line (BOI) of cytoplasmic male sterile

(CMS) *B. oleracea* var. *italica* (broccoli) as pollen receptor plants. The source of the

CMS in the *B. oleracea* inbred lines was the 'Anand' cytoplasm (Cardi and Earle

1997). These species were selected because they were among the highest value

Brassica vegetable seed produced. The imazamox resistant *B. napus* was not a

genetically modified organism (GMO) but provided a selectable marker that could be used to positively identify putative crosses. Seed from the glyphosate resistant canola, both *B. rapa* inbred lines, and leaf tissue from the *B. oleracea* inbreds was tested for the presence of the glyphosate-resistant trait with the Trait√[®] RUR test strip[8]. This kit detects the presence of the CP4 EPSPS protein produced by the *CP4 EPSPS* transgene which confers glyphosate-resistance. The testing was conducted both to ensure that no contamination of crossing stock existed prior to the experiment, and that the test was able to detect the protein in the transgenic canola.

Blue bottle flies (Diptera: Calliphoridae)[9] contained within 18,757 cm^3 mesh (18 x 16 mesh) cages were used to ensure pollen transfer (Currah and Ockendon 1984) and to exclude potential pollen transfer from other *Brassica* species. The *B. napus* x *B. rapa* crosses consisted of seven *B. rapa* receptor plants and three *B. napus* pollen donor plants. The *B. napus* x *B. oleracea* crosses consisted of three *B. oleracea* receptor plants and four *B. napus* pollen donor plants. The *B. oleracea* receptor plants were considerably larger than the *B. rapa* receptor plants, and thus there was only enough room to accommodate three in each mesh cage. Greenhouse conditions were set to provide a 20/20 C day night temperature with supplemental lighting to maintain a 14 h photoperiod. Both receptor plants and pollinator plants were fertilized once at the beginning of the experiment with Osmocote[®] 19-6-12 Smart Release® fertilizer[10], and were watered as needed.

Seed were harvested from individual receptor plants. Number of racemes, siliques, and seed per receptor plant was recorded for each cross. One hundred seed from each receptor plant, from each cross were placed in plastic germination boxes

containing moistened blotter paper[1] and placed into a germination chamber set to a

24/17 C day/night temperature regime with a 13 h photoperiod (Warman 1999). The

number of germinated seed was recorded for each receptor plant, and germination

percentages calculated for each cross. Non-germinating seed from the *B. napus* x *B.*

oleracea crosses were examined and tested for viability with a tetrazolium assay

according to methods described by the Association of Official Seed Analysts (AOSA

2000). Non-germinating seed from the glyphosate resistant *B. napus* crosses were

removed from the germination boxes and evaluated for the presence of the glyphosate-

resistant trait with the Trait√[®] RUR test strip.

Seedlings were removed from the germination boxes and transplanted into 28 x

53 cm flats filled with commercial potting soil[2] and grown in the greenhouse. At the

two leaf stage seedlings from each respective cross were treated with either 440 g ai

ha^{-1} glyphosate, or 183 g ai ha^{-1} imazamox plus a 90% non-ionic surfactant at 0.25%

v/v using a track sprayer calibrated to deliver 216 L ha^{-1} of spray solution (Warwick et

al. 2003). Plants were visually evaluated for necrosis 14 d after each herbicide

application, respectively. Plants surviving the herbicide application were scored as

hybrid individuals. For the glyphosate resistant crosses, surviving plants also were

tested with the Trait√[®] RUR strip to confirm resistance.

RESULTS AND CONCLUSIONS

Field Experiments. The number of seed produced on individual receptor plants varied

both by species and by year (Table 2-1). Germination also was variable among *B. rapa*

var. *chinensis* (BRCF) inbreds and averaged 29 and 58% in 2007 and 2008,

respectively. Viable seed were produced on the *B. rapa* var. *pekinensis* (BRPF)

inbreds in both 2008 and 2009, and averaged 62 and 64% germination, respectively.

Seed were only produced on the *B. oleracea* var. *italica* (BOI) plants in 2008;

however, they were shrunken and failed to germinate. Seed from the 2008 *B. oleracea*

var. *italica* x *B. napus* cross were tested for viability with a tetrazolium assay (AOSA

2002). Results of that assay determined that none of the seed contained viable

embryos. However, self fertilized seed produced on both of the cultivars of *B.*

oleracea var. *capitata* x *B. napus* were viable with an average germination of 81% for

inbred BOCF and 80% for inbred BOCM (Table 2-1).

Hybrids between *B. napus* (allotetraploid) and *B. rapa* or *B. oleracea* (both

diploid) were detected using flow cytometry analysis (Figure 2-1). Flow cytometry

analysis on the progeny produced from the *B. rapa* var. *chinensis* (BRCF) x *B. napus*

cross revealed that 74 and 89% of the offspring produced in 2007 and 2008,

respectively, were hybrids. Flow cytometry analysis on the progeny produced from the

B. rapa var. *pekinensis* (BRPF) x *B. napus* cross revealed that 17% and 15% of the

offspring produced were hybrids, in 2008 and 2009 respectively (Table 2-1). Although

hybridization rates varied between receptor plants (22% to 100%), there was a

relatively high outcrossing potential between these species in the field. None of the

progeny produced from the *B. oleracea* var. *capitata* (BOCF,BOCM) x *B. napus* were hybrids.

Tetraploid individuals were identified by flow cytometry in *B. rapa* x *B. napus* field crosses with the exception of those in 2008. Individuals deemed tetraploid were re-sampled and a second flow analysis conducted to confirm the results. While this analysis alone does not provide paternal identity, it does allow for some insights. Several other *Brassica* species including *B. nigra* and *Sinapsis alba* were present in small numbers at the field locations. However, all of these species are diploid. Therefore, a cross of one of the *B. rapa* receptor plants and one of these individuals would produce a diploid offspring, not a tetraploid. Since these individuals were confirmed as tetraploid in a second flow cytometry analysis, and they cannot be the product of outcrossing with any of the other compatible species, another mechanism, such as self fertilization of unreduced gametes (Heyn 1977) or somatic cell doubling, could likely be a responsible for this result. These mechanisms have been previously documented in *Brassicaceae*.

Identification of hybrid individuals using morphological characteristics was in agreement with the results of the flow cytometry analysis (data not shown). Diploid progeny of both *B. rapa* and *B. oleracea* displayed leaf, stem and floral characteristics that were identical to the maternal parent. Triploid progeny displayed a mixture of characteristics of the *B. rapa* and *B. napus* parents. While their acropetal leaves were similar in shape to the diploid parent, these individuals were easily identified by the presence hairs, blue green leaf color, and the corrugated stem characteristics of *B. napus*. Tetraploid progeny displayed a unique morphology closely resembling the

diploid parent, and lacking any of the characteristics of *B. napus* such as hairy leaves, or corrugated stems. These individuals did differ from the diploid progeny in that they were darker in color, and had a more segmented leaf.

The molecular marker screening confirmed the results of both the flow cytometry and the morphological analysis (Figure 2-2). Amplification of a DNA fragment corresponding to the A genome from *B. napus* was observed in hybrid progeny from both the *B. rapa* var. *chinensis* (BRCF) x *B. napus* (Figure 2-3) and the *B. rapa* var. *pekinensis* (BRPF) x *B. napus* crosses (Figure 2-4). No amplification was observed in any of the progeny of the *B. oleracea x B. napus* crosses confirming the identity of those crosses as self fertilization events.

Progeny from the *B. rapa* var. *chinensis* (BRCF) and *B. rapa* var. *pekinensis* (BRPF) which were determined to be tetraploid in the flow cytometry analysis did not display amplification of the A genome from *B. napus*. While the results of this study do not permit an explanation for the presence of the tetraploid individuals, they do allow us to say that they are not the result of hybridization with *B. napus*.

Greenhouse Experiments.

B. napus x *B. rapa* Crosses. The number of siliques, seed, and resulting germination of the seed varied greatly between both individual receptor plants and between inbred lines (Figure 2-5). While each of these crossing experiments were originally constructed using seven individual receptor plants, one of the BRCM inbreds died before seed set occurred.

The average number of siliques produced on inbred BRCM receptor plants was 101, while those of inbred line BRCF averaged 173. BRCM inbreds produced a greater amount of seed, averaging of 324 seed per plant, while those from BRCF inbreds produced an average of 95 seeds per plant. Germination of the seed produced by BRCM inbreds ranged from 88 to 98% among the individual receptor plants, and averaged 93%. While in the seed produced by inbred BRCF, germination ranged from 40 to 90% among the individual receptor plant, and averaged 73%.

The rate of hybridization with the glyphosate resistant canola also differed considerably between the inbred lines. No hybridization was observed between *B. napus* and BRCM inbreds. The hybridization rate between the glyphosate resistant *B. napus* and BRCF inbreds was 15.3% (Table 2-3).

In the crosses with the imazamox resistant *B. napus* (IMI), the number of siliques, seed, and resulting germination of the seed varied greatly between individual receptor plants and between inbred lines (Figure 2-5). The average number of siliques produced on BRCM inbred receptor plants was 148, while average number of siliques produced on BRCF inbreds was 349. Seed production also was greater on BRCF inbreds. BRCF inbreds produced an average of 224 seed per plant, while the BRCM inbred line averaged 371 seed per plant. Germination of the seed produced by BRCM inbreds ranged from 65 to 95% among the individual receptor plants, and averaged 83%. Germination of the seed produced by the BRCF inbreds averaged 87%, and ranged from 56 to 98% among the individual receptor plants.

The disparity in hybridization rate between cultivars was not as great in the imazamox resistant crosses as it was in the glyphosate resistant crosses. Seed produced

on BRCM receptor plants displayed a hybridization rate of 0.21%. However, BRCF

inbreds once again displayed greater hybridization with 0.9% of the screened

individuals surviving the herbicide application (Table 2-4).

Positive identification of the CP4 EPSP protein, which confers resistance to the

herbicide glyphosate, was detected in shrunken, non-germinating seed produced on the

conventional *B. rapa* plants. While the majority of the testing was performed with

three seeds per test, detection of the protein was possible using only one seed.

Additionally, some shrunken seed not used in the germination experiment were tested

and positive identification of the protein was detected. This result demonstrates that

fertilization did occur and at some point was terminated. Despite the abortion of the

embryo, cellular activity continued long enough to produce detectable levels of the

transgenic protein.

B. napus x B. oleracea Crosses. Visual observations of pollinator visits to the *B.*

oleracea flowers, and development of siliques indicated crossing potential. However

after harvesting the siliques, all of the seed produced from both the glyphosate and

imazamox resistant *B. napus* x *B. oleracea* crosses were shrunken and failed to

germinate. The total number of seed produced in the imazamox resistant *B. napus*

(IMI) x BOI inbred cross was 240, while the total number of seed produced in the

glyphosate resistant *B. napus* (RR) x BOI inbred cross was 130. Due to such low seed

production, the majority of seed produced in the glyphosate resistant *B. napus* (RR) x

BOI inbred cross was consumed in the germination testing. Therefore, tetrazolium

assays were performed on a total of 60 shrunken seed, 10 from each *B. oleracea*

receptor plant in each cross. The results of these assays determined that no embryo formation had occurred in any of the seed, indicating a lack of compatibility between these species. When the shrunken seed were tested for the presence of the CP4 EPSP protein using the Trait $\sqrt{}$® system, none of the shrunken seed tested positive for the presence of the protein. Unlike the aborted seed from *B. rapa* crosses, no paternal genetic material reached the ovaries; therefore, no transgenic protein was present in the aborted seed.

Implications. To our knowledge this is the first time crossing experiment of this kind have been conducted with inbred Brassica vegetable seed lines and canola. Results of these crossing experiments confirm that, although highly variable, interfertility does exist between *B. napus* and these modern *B. rapa* vegetable inbreds. Therefore, concerns about these species hybridizing are justified. The majority of the European cabbage, Chinese cabbage and other oriental *Brassica* vegetable seed crops grown in Oregon are produced for foreign, predominantly Asian, markets. These international purchasers of the vegetable seed crops have extremely low tolerances for any contamination. Contract requirements require that a seed lot be rejected if more than three outcrossed seed per 1,000 seed are found (Tichinin 2007). The majority of *Brassica* vegetable seed fields are small, usually less than 4 ha. Therefore, the large scale introduction of conventional canola into this production environment would serve as a large pollen source and could likely lead to the creation of undesirable off-types.

Detection of the CP4 EPSPS protein produced by the *CP4 EPSPS* transgene in shrunken seed demonstrates the potential to contaminate seedlots through adventitious presence. We believe this is first documented instance of this occurrence as it has not been mentioned previously in the literature. This finding may have serious implications for seed crops destined for markets with zero tolerance for transgene contamination. While most of the shrunken seed would likely be removed during seed cleaning, these aborted seed could serve as a contaminant. Many of the countries purchasing this seed have a zero tolerance for transgenic contamination; so, there is potential for the loss of the international markets if contamination is detected.

It is important to note that the data on hybridization presented here are limited to only the inbreds examined and should not be considered conclusive for all *B. rapa* or *B. oleracea* inbred lines. However, the results obtained in this study can be considered very robust for the species studied. Additionally, as was observed with *B. rapa* in the greenhouse experiments, inbred lines of the same species may differ greatly in their interfertility with canola. The results of these crosses show that hybridization rate is also dependant on the *B. napus* variety used in the cross. Therefore, the hybridization potential needs to be evaluated on a case by case basis.

These experiments in this study did not address how distance between *B. napus* and related *Brassica* vegetable seed fields might influence hybridization. Future work on this issue should focus on how isolation distance may impact the rate of outcrossing. Further research is also required to determine if similar results would be observed in other *Brassica* vegetable seed crops that share genomes with canola. Other vegetables in the *oleracea* genus such as kohlrabi, cauliflower, Brussels sprouts, and

kale also share the C genome with canola so may or may not prove to be as incompatible as the broccoli used in this study. Turnip, mizuna, and broccoli raab, all vegetable species in the *rapa* genus, which share the A genome with canola may not prove to be as compatible with *B. napus* as the Chinese cabbage we studied.

ACKNOWLEDGEMENTS

Table 2-1. Number of seeds produced, percent germination, and ploidy level of the plants as determined by flow cytometry of the in-field crosses conducted between the *Brassica* vegetable species and *B. napus*.

Year	Cross	Seed produced	Germination	Ploidy		
		#	%	2N	3N*	4N
				-------%-------		
2007	*B. oleracea* var. *italica* (BOI) *x B. napus*	0	--	--	--	--
2007	*B. rapa* var. *chinensis* (BRCF) *x B. napus*	16,620	29	23	74	3
2008	*B. oleracea* var. *italica* (BOI) *x B. napus*	10	0	--	--	--
2008	*B. rapa* var. *chinensis* (BRCF) *x B. napus*	4,999	58	11	89	0
2008	*B. rapa* var. *pekinensis* (BRPF) *x B. napus*	8,613	62	78	17	5
2009	*B. oleracea* var. *capitata* (BOCF) *x B. napus*	90	81	100	0	0
2009	*B. oleracea* var. *capitata* (BOCM) *x B. napus*	90	80	100	0	0
2009	*B. rapa* var. *pekinensis* (BRPF) *x B. napus*	911	64	84	15	1

* 3N (triploid) individuals are hybridization events between the receptor species and *B. napus*.

Table 2-2. The number of progeny by cross and year used in the morphological, flow cytometry, and molecular analysis screening.

Year	Cross	Individuals used in the screening #
2007	*B. oleracea* var. *italica* (BOI) *x B. napus*	0
2007	*B. rapa* var. *chinensis* (BRCF) *x B. napus*	135
2008	*B. oleracea* var. *italica* (BOI) *x B. napus*	0
2008	*B. rapa* var. *chinensis* (BRCF) *x B. napus*	73
2008	*B. rapa* var. *pekinensis* (BRPF) *x B. napus*	133
2009	*B. oleracea* var. *capitata* (BOCF) *x B. napus*	43
2009	*B. oleracea* var. *capitata* (BOCM) *x B. napus*	20
2009	*B. rapa* var. *pekinensis* (BRPF) *x B. napus*	204

Table 2-3. Number of individual seedlings used in the herbicide screening, survivors, and % hybridization from each of the receptor plants (1-7) in the glyphosate resistant *B. napus* (RR) x *B. rapa* greenhouse crossing experiments.

B. rapa inbred line	Number of treated seedlings	Number of survivors	% Hybridization
BRCM			
(1)	59	0	0
(2)	25	0	0
(3)	56	0	0
(4)	53	0	0
(5)	51	0	0
(6)	65	0	0
(7)	61	0	0
BRCF			
(1)	23	9	39
(2)	17	6	35
(3)	21	7	33
(4)	18	3	17
(5)	2	0	0
(6)	17	2	12
(7)	0	0	0

Table 2-4. Number of individual seedlings used in the herbicide screening, survivors, and % hybridization from each of the receptor plants (1-7) in the imazamox resistant *B. napus* (Imi) x *B. rapa* greenhouse crossing experiments.

B. rapa inbred line	Number of treated seedlings	Number of survivors	% Hybridization
BRCM			
(1)	55	0	0
(2)	64	0	0
(3)	37	0	0
(4)	51	1	2
(5)	52	0	0
(6)	62	0	0
(7)	61	0	0
BRCF			
(1)	41	0	0
(2)	29	0	0
(3)	22	0	0
(4)	1	0	0
(5)	10	1	10
(6)	7	0	0
(7)	0	0	0
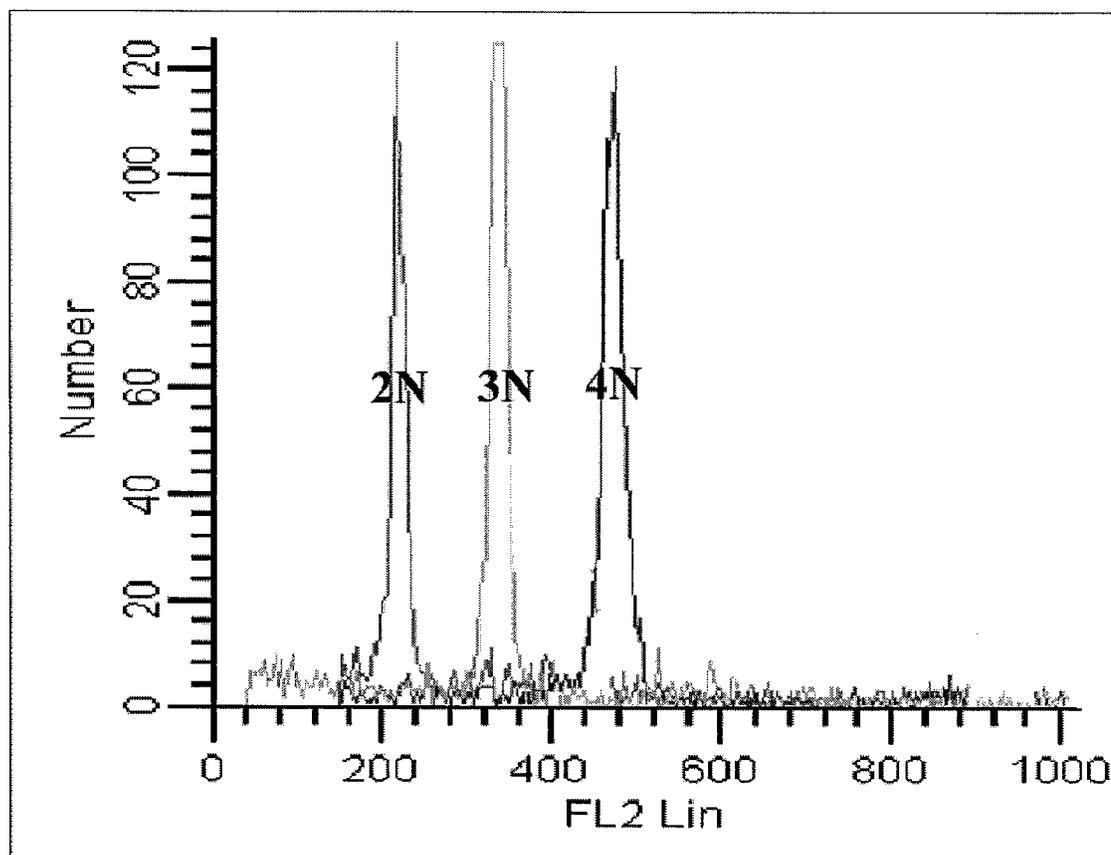
Figure 2-1. Flow cytometry peaks delimitating the triploid (3N) hybrid individuals from the diploid (2N) *B. rapa* (BRCF) and the tetraploid (4N) *B. napus* parental species. Resolved on a FL2 linear scale at 639 volts.
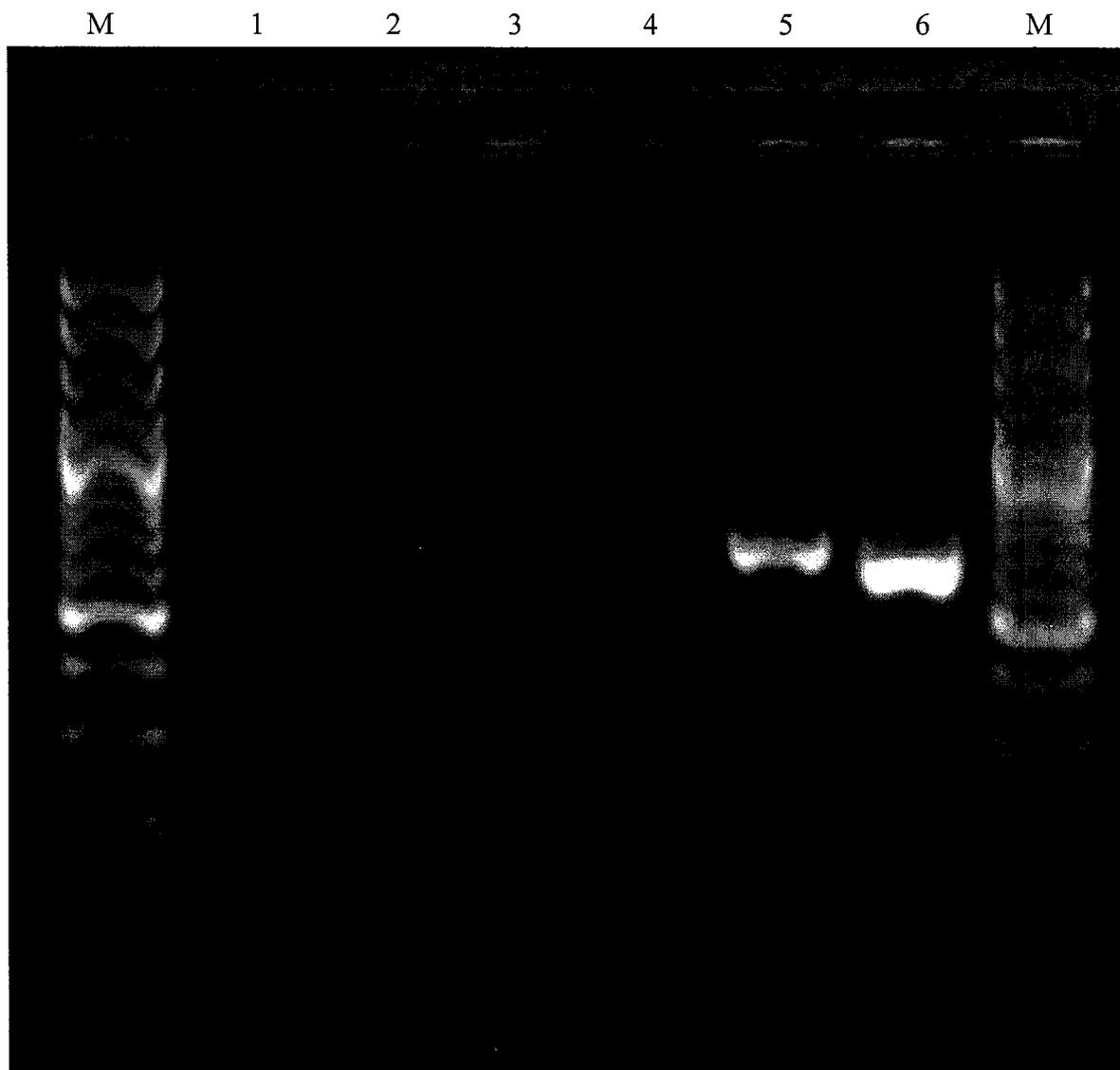
Figure 2-2. Marker profile for primer set 7 showing no amplification in either the *B. rapa* var. *chinensis* (BRCF) in bred line in Lanes 1-2, or *B. rapa* var. *pekinensis* (BRPF) inbred line in Lanes 3-4, and amplification (680 bp) of the A genome from *B. napus* in Lanes 5 and 6.

33
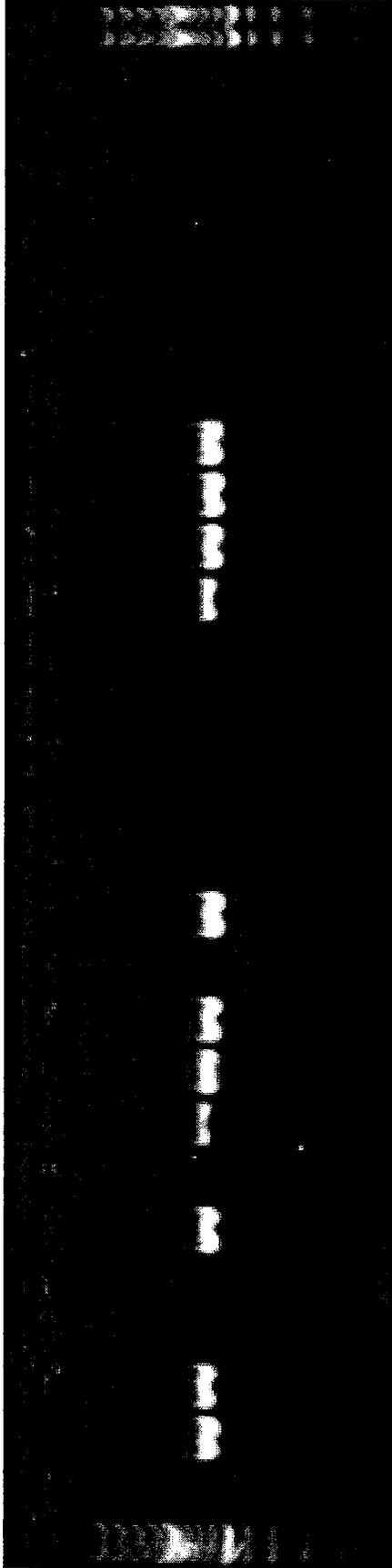
Figure 2-3. Molecular marker profile for primer 7 showing amplification (680 bp) of the A genome from *B. napus*, indicating a positive hybridization. Lanes 1-21 are offspring from the *B. rapa* var. *chinensis* (BRCF) x *B. napus* cross. Lanes 22-28 are offspring from the *B. oleracea* var. *capitata* (BOCF) x *B. napus* cross.
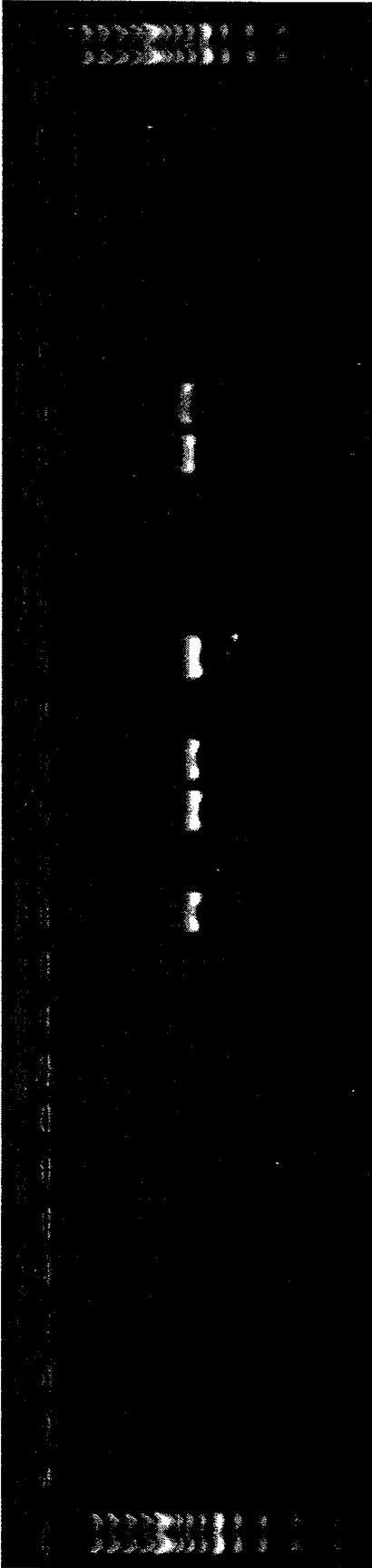
Figure 2-4. Molecular marker profile for primer 7 showing amplification (680 bp) of the A genome from *B. napus*, indicating a positive hybridization. Lanes 1-11 are offspring from the *B. oleracea* var. *capitata* (BOCM) x *B. napus* cross. Lanes 12-28 are offspring from the *B. rapa* var. *pekinensis* (BRPF) x *B. napus* cross.
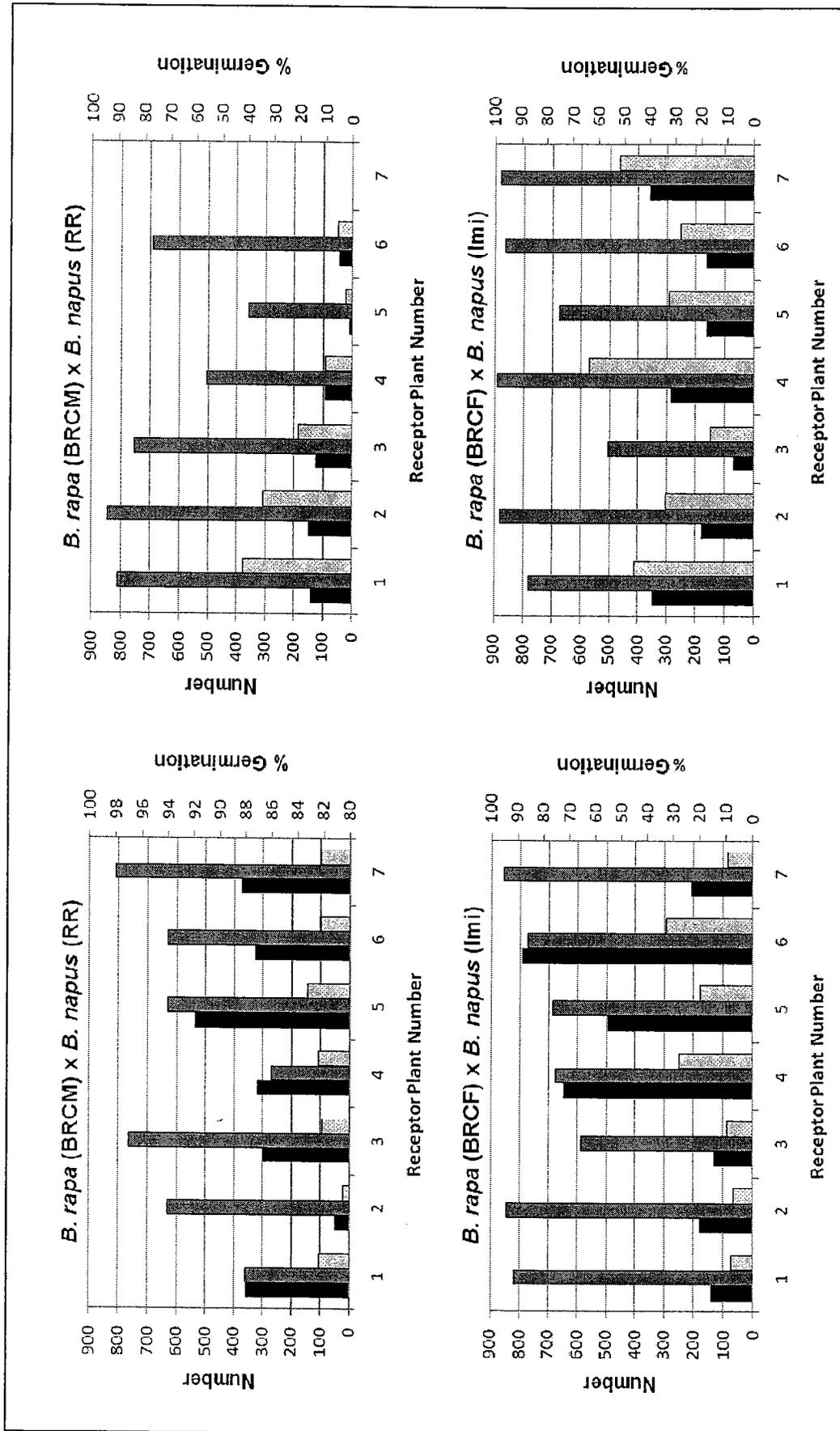
Figure 2-5. Number of seed (■), percent germination of the seed (■), and number of siliques (□) by receptor plant, from each of the glyphosate resistant (RR) or imazamox resistant (Imi) *B. napus* x *B. rapa* greenhouse crossing experiments.

SOURCES OF MATERIALS

[1] BB44 Steel Blue Blotter, Hoffman Manufacturing Inc, Albany, OR 97321.

[2] Sunshine Mix #4. Sun Gro Horticultural Distribution Inc. Bellevue, Washington 98008.

[3] Beckman Coulter FC 500 Flow Cytometer, system ID# 445872. Beckman Coulter Inc. Brea, California 92822.

[4] CXP Analytical Software System. Beckman Coulter Inc. Brea, California 92822.

[5] DNeasy® 96 Kit, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355

[6] Taq DNA Polymerase, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355

[7] C1000™ Thermal Cycler, Bio-Rad, 2000 Alfred Nobel Drive Hercules, CA 94547

[8] Trait√® RUR test strip. Strategic Diagnostics Inc, Newark, DE 19702.

[9] Blue Bottle Flies (Ref: TSU Lot #96-8464). Forked Tree Ranch, Bonners Ferry, ID 83805.

[10] Osmacote® Smart Release® Plant Food. Scotts-Sierra Horticultural Products Co, Marysville, OH 43041.

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CHAPTER 3: IN FIELD ASSESSMENT OF CANOLA (*Brassica napus* L.) SEED PERSISTANCE AND VOLUNTEER POTENTIAL IN THE WILLAMETTE VALLEY OF OREGON

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ABSTRACT

Canola (*Brassica napus* L.) is an economically important crop grown worldwide and produces high seed and oil yields. The use of canola oil for biodiesel production has greatly increased the demand for this crop. This increased demand has consequently created interest in growing canola in areas outside of its traditional production range, sometimes causing conflict between canola growers and growers of traditional crops in the those areas. Producers of vegetable *Brassica* seeds voiced concerns about the potential negative impacts, such as seed contamination due to outcrossing, that planting larges acreages of canola might have on their industry in the Willamette Valley in Oregon. Canola seed is capable of persisting in soil, creating volunteer plants in successive crops. Most studies addressing this seed persistence issue have been conducted in Canada or the UK, both of which have climates very different from that of the Willamette Valley. Field experiments were conducted in order to assess the seed persistence and volunteer potential of canola in the Willamette Valley. Five field sites, never previously planted with canola, were studied for three years following an initial canola planting. Twenty soil cores were taken from each of the field sites each year, the seed extracted and tested for germination to monitor seed persistence. Following canola harvest, shattered seed were collected from both harvested windrow and non-windrow locations in 0.25 m^2 quadrats along 30 m transects randomly distributed in the fields. Approximately 30 days after these samples were taken, seedling recruitment was quantified in quadrats placed immediately adjacent to the mapped location of the shatter samples. Canola seed were

recovered from soil cores taken at each of the field site. Germinable seed was found at two locations, but only in one year. In-field measurement of postharvest seed losses varied between windrow and non-windrow locations, and between fields and years. Volunteer canola assessments also indicated differences between windrow and non-windrow locations, and between grower locations and years. Visual assessment of the grower fields in the years following the initial canola planting detected volunteer plants at two of the field locations. However, these plants were controlled prior to flowering. No further volunteer plants were observed at any of the field sites. Canola seed is capable of persisting in the soils of the Willamette Valley; however, grower implemented control practices appear to be sufficient to control and suppress volunteer plants in the field.

Nomenclature: canola, *Brassica napus* L.

Key Words: seed persistence, volunteer, shatter, vegetable seed.

INTRODUCTION

Canola (*Brassica napus* L.) seed is capable of persisting in the soil for long

periods of time (Gruber et al. 2004). Some studies have documented survival of canola

seed for up to 10 years in undisturbed soil (Schlink 1998). Additionally, there is

evidence that in semi-natural habitats, such as roadway ditches, canola can persist for

a similar period of time (Pessel et al. 2001). Legere et al. (2001) reported that the seed

will survive in the soil seedbank at least four years after the crop was grown in studies

conducted in Canada.

Management practices greatly impact the persistence of canola seed (Hails et al.

1997). Seed left on the soil surface typically germinate and the resulting seedlings can

be removed by tillage or chemical control (Lutman et al. 2003). However if the seed is

buried, it has the ability to undergo secondary seed dormancy and survive much longer

(Pekrun and Lutman 1998). Burial permits the seed to survive more than one season,

producing a persistent seedbank. Secondary seed dormancy in canola varies

considerably by genotype (Gulden et al. 2003b). Gruber et al. (2004) found that seed

persistence varied from 7 to 90% depending upon cultivar over a two year period.

Seed losses during harvest can range from 3 to 10% depending on harvest method

and environmental conditions at harvest. This seedbank input represents 9 to 56 times

the normal seeding rate of a canola crop (Gulden et al. 2003a, 2003b). The same

authors reported an average of 107 kg per ha seed loss for 35 fields sampled. There are

250,000 to 300,000 canola seeds per kilogram, so it is possible that there were could

be 2.7 million seeds ha^{-1} returned to these canola fields. Densities of 2,000 and 10,000

seeds m^{-2}, respectively, were reported left on the field after harvest in Canada and the

UK (Legere et al. 2001; Lutman 1993). Harvest losses of 6000 seeds m^{-2} are considered to be typical in Denmark (Lutman and Lopez-Granados, 1998).

Volunteer canola can be a significant weed management problem in subsequent crops (Kaminski 2001). Although canola that escapes from cultivation does not generally survive in undisturbed habitats, it can survive in areas adjacent to agricultural sites, such as roadsides and field edges (Beckie et al. 2000; Schafer et al. 2010; Warwick et al. 1999). Volunteer canola plants can act as a bridge for insect and disease pests between rotations (Gruber et al. 2004). Additionally, herbicide resistant varieties of canola will produce herbicide resistant volunteer plants. While these plants may complicate chemical control strategies, they also serve as a source for gene dispersal in time and space via pollen and seed movement (Pekrun et al. 2005, Hall et al. 2000).

Western Oregon, with its mild winters and dry, summers has the ideal climate for the production of seed crops. In the Willamette Valley in Oregon, the specialty seed crop industry produces vegetable and flower seeds. While the *Brassica* specialty seed crop growing area is small, it is very profitable, often netting a grower more than $4,000 per hectare (Ehrensing 2007). In fact, western Washington and Oregon combined produce nearly all (~90%) of the global supply of European cabbage (*B. oleracea* var. *capitata*), Brussels sprouts (*B. rapa* var. *gemmiferae*), rutabaga (*B. napus* var. *napobrassica*) and turnip (*B. rapa* var. *rapifera*) seed, and a substantial portion (20 – 30 %) of radish (*Raphanus sativus*), Chinese cabbage (*B. rapa* var. *chinensis*) and other oriental *Brassica* vegetable crops (Myers 2007). Fifty to 60% of

the seed is exported to Europe and Asia, constituting a significant portion of the global *Brassica* vegetable seed market.

This high value *Brassica* vegetable seed crop production could be jeopardized if contamination occurs from canola hybridization with vegetable varieties. The risk of market loss is even greater if the crops are contaminated with transgenic canola. International purchasers of the vegetable seed crops have extremely low tolerances for any contamination and some maintain a zero tolerance for transgenic contamination (Tichinin 2007). Transgenic canola was introduced in Canada in 1995 and was overwhelmingly accepted by growers. Only 10 years after the introduction, in 2005, 82% of the canola produced was transgenic (Beckie et al. 2006). Currently, transgenic canola comprises 86% of the crop grown in Canada (Beckie and Warwick 2010). The specialty seed crop industry is fearful that if canola for oilseed production is allowed in Willamette Valley the same trend might occur in Oregon.

The majority of canola seedbank studies have been conducted in the Northern Great Plains of the U.S., in Canada, or Europe (Gulden et al. 2003a, 2003b; Legere et al. 2001; Lutman 1993). The climates of these regions differ from the Mediterranean climate of the Willamette Valley. The Northern Great Plains of the U.S. and Canada are classified as Hemiboreal climates and experience summers that are often wetter than winters. While Maritime climates with cool, rainy summers dominant European canola growing regions (Peel et al. 2007). Canola seed persistence data and the resulting volunteer potential in climates such as those of the Pacific Northwest are lacking in the literature. Therefore, the objective of this study was to quantify canola

seed persistence and potential to volunteer under the agronomic and environmental conditions of the Willamette Valley.

MATERIALS AND METHODS

Seed persistence. Five commercial field sites located in the Willamette Valley of Oregon were monitored for three years following an initial canola planting at the commercial sowing rate (~9 kg/ha) (Table 3-1). The field sites ranging in size from 6 to 16 ha were selected because they had never been previously planted with canola. Twenty soil cores 8.3-cm in diameter and 10-cm deep were taken in a "W" pattern in each of the fields each year (Baker and Preston 2008; Harker et al. 2006). Soil samples were taken the last week of March, prior to canola flowering, to establish a baseline seedbank estimate in the first year. Subsequent soil samples were then taken at each of the sites, always in the last week of March, for the next two years. The soil cores were stored at 5 C prior to prevent canola seed germination and microbial decay before the samples could be analyzed.

Shortly after sampling, direct extraction of seeds from the soil was accomplished with elutriation methods protocols previously established for seedbank studies. This method consisted of two stages (adapted from Wiles et al. 1996). The soil samples were sieved in the first stage through three Tyler[1] grading screens stacked in descending order: 1/15 mesh (top), 1/18 mesh (middle), 1/19 mesh (bottom); and washed with a high pressure water stream with hand agitation until all large soil

particles were removed. Remaining portions of the sample were collected from the 1/18 and 1/19 screens and bulked in a plastic bag and stored at 5 C.

Samples were re-suspended in water and agitated lightly by hand in the second stage of the process. Excess water was then removed through a Tyler[1] screen 42 mesh. This process was repeated three times per sample. Samples were allowed to air dry for 24 h at room temperature. Next, each sample was examined under a stereoscopic microscope[2]. Any putative canola seed found was removed from the sample and placed in plastic germination boxes containing moistened blotter paper[3] and placed into a germination chamber set to a 24/17 C day/night temperature regime with a 13 h photoperiod (Warman 1999) and any germinated seed counted. The remainder of each sample was spread over 28 x 53 cm flats filled with commercial potting soil[4] and placed in a greenhouse set to maintain a 20/20 C day/night temperature without supplemental lighting. The flats were visually evaluated for 30 d for canola plant emergence to ensure no canola seed were missed in the visual assay.

Seed loss from shattering. Sampling of shattered seed loss was conducted over four site-years, Site 1 and Site 3 in 2007, and Site 4 and Site 5 in 2008 immediately after harvest (Table 3-1). Site 2 had poor canola emergence and was terminated by the cooperator; therefore, Site 2 was only used in the seed persistence study. Four 30 m transects were laid perpendicular to the windrows at random locations in each field. Samples from 25 x 25 cm quadrats at 1 m intervals along each transect were collected within and between harvested windrows using a wet-dry vacuum cleaner[5] (Gulden 2003). Windrow locations were readily determinable as the stubble under the windrow

was distinctly less weathered than stubble not under the windrow. The location of each sample was mapped using a handheld GPS device[6]. Samples were cleaned of soil, weighed and the seed per unit area calculated based on 1000 seed weights.

Volunteer assessment. Approximately 30 days after the seed shatter samples were taken, canola seedling recruitment counts were made, using the same protocol as the seed shatter sampling, in quadrats placed immediately adjacent to the mapped location of the seed shatter samples. Additionally, each of the field sites and borders were visually monitored through multiple site visits for volunteer canola plants for three years following the initial planting.

Due to the variability in the number of shattered seed, and volunteer number per sample, mean values for each transect were used in the analysis. Additionally, individual samples (n=3) with values greater than 5 standard deviations from the sample mean were considered outliers, and dropped from the sample set (Kling 2010).

Statistical analysis was conducted using PROC GLM in SAS v9.1 (SAS 2002). Means comparisons, and t-tests were conducted for both the seed shatter and volunteer canola seedling data using the MEANS procedure.

RESULTS AND CONCLUSIONS

Seed persistence. Seed were recovered from Sites 1, 2 and 3 in 2007. However, in

2008, no seed were recovered from either of the sites planted that year. Canola seed were recovered from the soil samples taken from Sites 4 and 5 the following year (Table 3-2). Wide fluctuations in environmental conditions between years and sites, unsuccessful germination or establishment, or varying levels of predation can result in the rapid decline of viable canola seeds on or near the soil surface (Gulden et al. 2003b; Legere 2001). The influence of these factors can produce a wide variation in seedbank contribution among field locations, and between years at the same location. Additionally, variation in recovered seed could have been impacted by the sampling methods used and may not reflect seedbank fluctuations.

Following harvest at each of the sites, with the exception of Site 2, shattered seed was left on the soil surface and allowed to sprout, then the resulting volunteers controlled by chemical and cultural means (Table 3-2). Site 2, however, was moldboard plowed after poor emergence and frost damage resulted in a poor canola stand.

In 2007 and 2008, none of the seed recovered from the soil samples germinated. While in 2009, one seed from Site 3, and one seed from Site 4 did germinate in the growth chamber (Table 3-2). The soil sampling conducted in 2009 encompassed the last year of field sampling for Sites 1-3, and the second year of sampling for Site 4. Interestingly, this indicates that at Site 3 viable seed had persisted in the soil for at least one year prior to the sampling without being detected. Since any volunteers present at this location were controlled and not allowed to flower, this seed would have entered the seedbank either with the initial planting, or following harvest. The 2009 soil sampling at Site 4 was conducted only 1 year following canola

production at that site. Therefore the viable seed that were recovered from this site also persisted in the soil for at least 1 year. While viable seed were not found at either Sites 1 or 2, they may have been missed during sampling. None of the seed recovered from 2010 soil samples germinated. These results demonstrate that viable canola seed is capable of persisting in Willamette Valley cropping environments.

Seed loss from shattering. Harvest and shatter sampling dates for each field site are listed in Table 3-1. The in-field shatter counts differed in seed shatter between harvested windrow and non-windrow locations within the same field, with the exception of Site 3 (Figure 3-1). The total amount of shattered seed also varied considerably by location. This difference in amount of shattered seed between locations may be attributable to grower experience as this was the first time some of them had produced canola.

The yields, harvest loss, and percentage of yield loss due to shatter for each of the field sites are shown in Table 3-3. The number of seed lost to shatter at these sites represents a potential return to the seedbank of 9 (71 kg/ha^{-1}) to 32 (260 kg/ha^{-1}) times the initial seeding rate. In a study conducted in Canada, Gulden et al. (2003a) reported an average post harvest yield loss from ranged from 3.3 to 9.9% of total yield or approximately 9 to 56 times the normal seeding rate. In Europe harvest losses of 600 kg/ha^{-1} or 25% of recorded yield are not uncommon (Price et al. 1996; Hobson and Bruce 2002). While this was the first experience growing canola for several of the cooperating growers in this study, over all their harvest losses are comparable with those of more experienced growers.

Volunteer persistence. The individual sampling dates for the volunteer assessments at each field location are listed in Table 3-1. There were differences in the number of field volunteers between each of the field sites (Figure 3-2). Differences in the mean number of volunteers between harvested windrow and non-windrow locations within the same field were only observed at Sites 4 and 5.

Following grower implemented management practices (Table 3-2), volunteers were not detected in any of the monitored field sites in the following crops as of the 2008 growing season. However, in the late fall of 2009 a considerable number (> 50) of volunteers were observed at Site 3 (planted to perennial ryegrass) and Site 5 (planted to wheat) field locations. Subsequent visual observation of these sites through the 2010 growing season did not detect any further volunteer plants at any of the field sites. Because volunteer canola is relatively easy to control in subsequent monocot crop rotations, in-field volunteers are not expected to be problematic (Begg et al. 2006).

Conclusions. Viable canola seed is capable of persisting in soils and environment of the Willamette Valley. A great deal of canola seed is lost due to shatter, but when left on the soil surface, the majority of this seed may germinate and seedlings can be controlled (Gruber et al. 2004). Volunteer plant assessments in this study were only conducted once and do not account for any subsequent germination of shattered seed after the counts were taken. One of the cooperating growers noted that there were several flushes of germination of the shattered seed (Freeborn 2009). This may explain the disparity between the number of recovered seed in the soil cores and the estimated

several flushes of germination of the shattered seed (Freeborn 2009). This may explain the disparity between the number of recovered seed in the soil cores and the estimated number of seed returned to the seedbank from harvest loss. In a study conducted in England, Austria, and Germany delaying the incorporation of canola seed by leaving the stubble untouched for 4 wk resulted in a reduced canola seedbank (Pekrun et al. 2006).

Seed was discovered at Site 2 in the years following the initial planting, despite removal of the canola crop prior to flowering. The canola crop at this site was plowed with a moldboard plow, burying the seed and allowing it to survive. Studies conducted in Europe reported that canola harvest loss contribution to the seed bank was larger after inversion tillage by moldboard plow than after shallower tillage methods (Baker and Preston 2008; Gruber et al. 2005). Finally, while volunteer canola plants were noted, they were removed from the crop. Therefore, it is unlikely that volunteer persistence will be an issue within fields in monocot crop rotations commonly used in the Willamette Valley. However, rotations utilizing dicot crops following canola were not included in this study and may produce different results.

ACKNOWLEDGEMENTS

Table 3-1. Elevation, soil type, planting date, date of harvest, shattered seed sampling, and volunteer plant sampling of the five field locations.

Field Site	Elevation (m)	Soil type	Planting date	Harvest date	Sampling dates Shatter	Volunteer
1	74.4	Dayton-silt loam	9/27/2006	7/6/2007	7/18/2007	8/31/2007
2*	77.1	Coburg- silty clay loam	9/22/2006	---	---	---
3	83.5	Amity- fine silty loam	10/04/2006	7/6/2007	7/19/2007	8/30/2008
4	61.9	Woodburn-silt loam	9/25/2007	7/11/2008	8/1/2008	9/3/2008
5	84.1	Woodburn-silt loam	9/27/2007	7/15/2008	7/31/2008	9/2/2008

*Site 2 was removed from production due to poor canola emergence; therefore no harvest, shattered seed, or volunteer plant counts were possible.

Table 3-2. Presence (+) or absence (-) of canola seeds in soil cores taken from each field site, by year sampled.

Site	Estimated # of seed/ha furrow slice			
	2007	2008	2009	2010
1[†]	+	+	+	NA
2[†]	+	+[τ]	-	NA
3[†]	+	-	+[τ]	NA
4[*]	NA	-	+[τ]	+
5[*]	NA	-	+	+

[†] Studies concluded in 2009.
[*] Studies initiated in 2008.
[τ] Germinable seed.

Table 3-3. Yield and estimated harvest losses due to shatter at field locations.

Field site	Yield	Harvest loss	% Yield loss
	-------------------- kg ha^{-1} --------------------		
1	486	260	53
2[*]	NA	NA	NA
3	623	188	30
4	345	81	23
5	605	71	12

[*]Site 2 was removed from production due to poor canola emergence.

Table 3-4. Grower implemented volunteer management practices at each of the field sites, following canola harvest.

Field site	Grower management practices
1	Applied glyphosate @ 0.29 L ha^{-1} post canola seed sprouting, then no-till planted winter wheat. Single application of flufenacet + metribuzin @ 0.29 L ha^{-1} post wheat emergence in the spring.
2	Crop removed from due to poor emergence with glyphosate @ 0.29 L ha^{-1}, moldboard plowed before planting spring wheat, then winter fallowed.
3	Allowed canola seed to sprout then failed and flex harrowed prior to winter wheat planting. Single application of MCPA @ 1.17 L ha^{-1} post wheat emergence in the spring.
4	Allowed canola seed to sprout then moldboard plowed before planting winter wheat. Fall applied flufenacet + metribuzin @ 0.29 L ha^{-1} post wheat emergence.
5	Allowed canola seed to sprout then moldboard plowed before planting winter wheat. Fall applied flufenacet + metribuzin @ 0.29 L ha^{-1} post wheat emergence in the fall. Single application of MCPA + dicamba @ 1.17 L ha^{-1} in the spring.
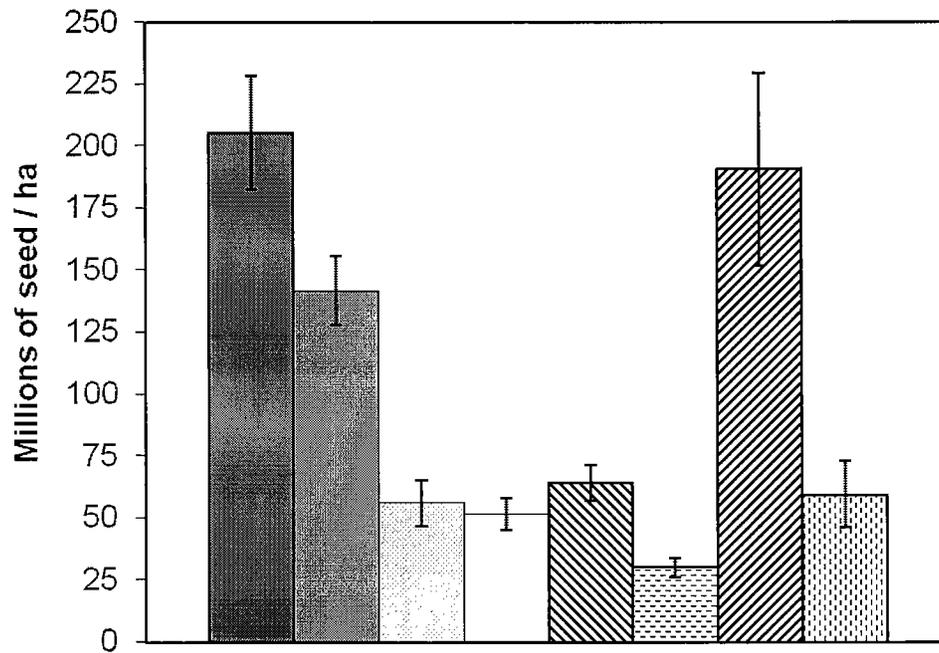
Figure 3-1. Number of shattered canola seeds from field locations: Site 1 windrow (▓), Site 1 outside the windrow (▓), Site 3 windrow (▓), Site 3 outside the windrow (☐), Site 4 windrow (▨), Site 4 outside the windrow (▤), Site 5 windrow (▨), Site 5 outside the windrow (▦). Error bars represent the standard errors of the mean of four transects.
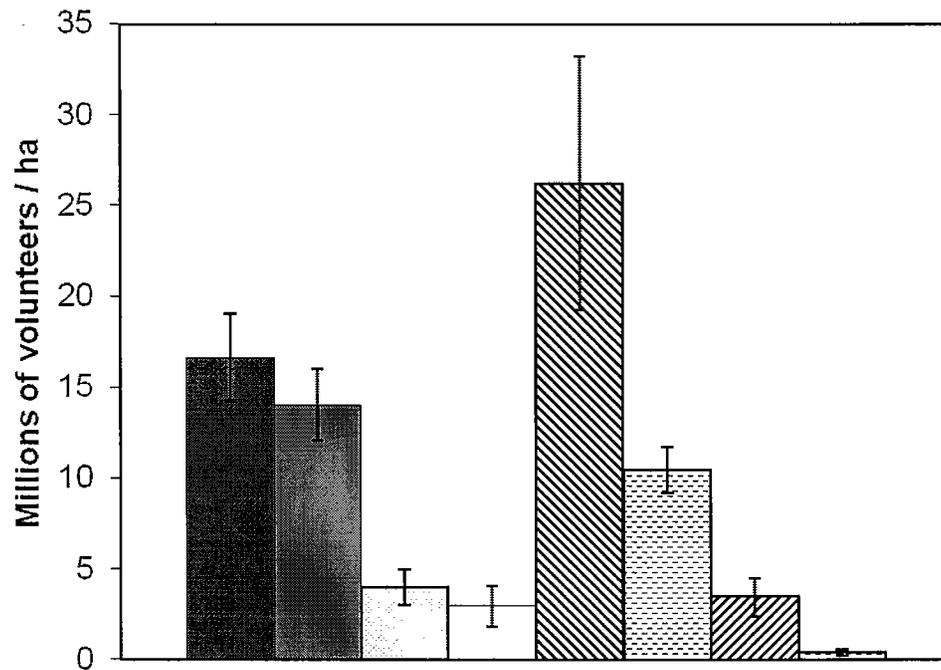
Figure 3-2. Number of canola volunteer plants from field locations: Site 1 windrow
(■), Site 1 outside the windrow (▦), Site 3 windrow (▨), Site 3 outside the
windrow (□), Site 4 windrow (▨), Site 4 outside the windrow (▤), Site 5 windrow
(▨), Site 5 outside the windrow (▨). Error bars represent the standard errors of the
mean of four transects.

SOURCES OF MATERIALS

[1] Tyler mesh sieves. Hoffman Manufacturing Inc, Albany, OR 97321.

[2] Digital stereoscope. Model # MP-MZD-1. Westover Scientific. Mill Creek, Washington 98012.

[3] BB44 Steel Blue Blotter, Hoffman Manufacturing Inc, Albany, OR 97321.

[4] Sunshine Mix #4. Sun Gro Horticulture Distribution Inc. Bellevue, Washington 98008.

[5] Wet/Dry vacuum cleaner. Model #3150. Shop-Vac Corporation. Williamsport, Pennsylvania 17701.

[6] eTrex Legend global positioning system. Garmin, Olathe, Kansas 66051.

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CHAPTER 4: GENERAL CONCLUSIONS

Often when conducting research, we are confronted with issues that are far too large in spatial and temporal scale to explore with the resources available. Such is the case with this study. The larger issue of how large scale planting of canola would impact the *Brassica* vegetable seed industry would, in truth, require many years and considerable acreage to study. Since these requirements are not feasible, we must find ways to evaluate some of the key factors on a scale that is manageable.

First, there is the issue of compatibility between the *Brassica* vegetable species and canola. General predictions of the hybridization potential of the various *Brassica* species as outlined by U (1935) were supported by our findings. As predicted, the *Brassica rapa* species did hybridize with canola under both field and greenhouse conditions. However, neither of the two *Brassica oleracea* species we examined produced any hybrids. The degree to which the hybridization varied depending upon what subspecies and inbred *B. rapa* were used in the cross. Greenhouse studies demonstrated that hybridization rate also is influenced by the *B. napus* cultivar used in the cross. These studies were conducted as a "worst case scenario" in terms of pollen load favoring hybrid production as each *Brassica* vegetable seed receptor plant was placed in the middle of a canola field during peak flowering. Therefore, if there was even low compatibility between the *Brassica* vegetable species and canola, we would obtain a hybrid. But if there were no hybrids produced, the likelihood of one occurring naturally would be exceedingly small. However, this brings to light another limitation inherent in this study. For these crosses, we used subspecies and cultivars supplied to us by the vegetable seed producers as economically important cultivars. But,

frequently these growers are given different cultivars each year. So, it is important to stress that the results we obtained can only be applied to the cultivars we tested.

Second, there is the issue of whether transgenes would be detectable in the harvested *Brassica* vegetable seed, even if it was not viable. In greenhouse crosses with *Brassica rapa* and genetically modified (GM) glyphosate resistant *B. napus,* we were able to obtain shrunken seed that, while not germinable, did test positive for the presence of the transgene. While most of the shrunken seed would likely be removed during seed cleaning, dust or residue from these aborted seed could serve as a contaminant. Some of the countries purchasing this seed have a zero tolerance for transgenic contamination; thus, there is potential for the loss of these international seed markets if contamination occurred.

Results of these crossing experiments confirm that, although highly variable, interfertility does exist between canola and these modern *B. rapa* vegetable cultivars. Therefore, concerns about these species hybridizing in the field are justified. The majority of the European cabbage, Chinese cabbage and other oriental *Brassica* vegetable seed crops grown in Oregon are produced for foreign, predominantly Asian, markets. These international purchasers of the vegetable seed crops have extremely low tolerances for any contamination. Contract requirements require that a seed lot be rejected if more than three outcrossed seed per 1,000 seed is found (Tichinin 2007). The majority of *Brassica* vegetable seed fields are small, usually less than 4 ha. Therefore, the large scale introduction of canola into this production environment would serve as a large pollen source and could likely lead to the creation of undesirable off-types.

Third, there was the issue of whether volunteer canola had the potential to become a contaminant in the *Brassica* vegetable seed crops. The approach we took was to measure canola seed loss due to shatter and monitor the volunteers produced. Additionally, we examined canola seed persistence in the soil of these fields to try to ascertain if canola would be a problem weed in the following crop rotations. We observed that there is a considerable amount of canola seed lost to shatter during harvest. This is especially true at locations where growers do not have much experience with the crop. However, if managed properly, canola does not appear to be a persistent weed in the following crop rotations. The successful management strategy hinges on leaving the shatter seed on the soil surface to sprout and then controlling the seedlings with chemical and cultural means. While we did observe that canola seed is capable of persisting in the soil seedbank, volunteers are easily controlled in the following monocot crop. Whether this would be the case following broadleaf crop was not examined in this study.

The ODA ruled on the establishment of rapeseed control district boundaries in 2009, under statute ORS 570.450, to protect the vegetable seed industry. This ruling states that canola may not be grown for the production of oil within this control area, with the exception of special permits issued for research. The control area designated in this ruling encompasses the majority of the fields in the Willamette Valley, severely curtailing the possibility of growing canola as a rotation crop in production fields west of the Cascades. However, the ruling also left provisions for a re-evaluation in 2012.

Provisions were also made to permit continued research during this three year period. This may allow for studies to address other key aspects, such as distance of

pollen movement, which we were unable to examine in our research. Currently, the vegetable seed producers maintain a three mile buffer zone around individual fields (Tichinin 2007). But this distance is based purely on speculation as no research has addressed the impact of pollen mediated gene flow in *Brassica* vegetable species.

Future studies should focus on how volunteers in non-crop areas, such as roadsides, might influence pollen mediated gene flow. While our findings indicate that, in well managed field, in-field volunteers likely do not pose a threat, those plants that are along transportation corridors, which are widely dispersed and allowed to flower, may. Additional crossing experiments need to be conducted and extended to other *Brassica* sub-species, such as *Raphanus sativus* that are grown for seed in the Willamette Valley, and its weedy relative *R. raphanistrus*.

This research should be viewed as the first steps into examining the larger issue of what the impact of planting large areas of canola would have on the *Brassica* vegetable seed industry in the Willamette Valley. Definitive answers are almost never present with issues that concern natural systems at a landscape scale. This research is no different. However, our results should be helpful in providing information to policy makers so that they can make decisions, based on factual evidence and not just conjecture.

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APPENDIX

APPENDIX A: ESTIMATING DISTANCE OF POLLEN MEDIATED GENE FLOW BETWEEN HERBICIDE RESISTANT CANOLA AND A RELATED BRASSICA VEGETABLE SPECIES

A preliminary study was conducted in 2009 to estimate the effective distance of pollen mediated gene flow between *Brassica napus* (canola) and *Brassic rapa* var. *chinensis*, a related species grown for vegetable seed. Approximately 0.2 ha at the Oregon State University Hyslop Experimental Farm was planted with 'Clearwater' canola, a variety resistant to the herbicide imazamox, at the commercial sowing rate (~ 9 kg/ha). No other herbicide resistant canola was grown on the farm or in the surrounding area that year. Eighteen *B. rapa* var. *chinensis* plants were grown in the greenhouse and moved into the field when the canola began flowering, and returned to greenhouse 15 d later. The *B. rapa* plants were distributed in a cross pattern centered on the canola planting, with both the east-west and north-south axis terminating at the bounds of the research farm (Figure A-1). The location of each *B. rapa* plant was recorded with a GPS unit. Seed of each receptor plant were harvested individually. Number of racemes, siliques, and seed per receptor plant was recorded for each cross. All seeds from each of the receptor plants were planted into 28 x 53 cm flats filled with commercial potting soil and grown in the greenhouse set a 20/20 C day/ night temperature and no supplemental lighting. Additionally, seeds of the *B. rapa* var. *chinensis* were planted for use as a control in the herbicide screening.

At the two leaf stage seedlings were treated with 183 g ai ha^{-1} imazamox plus a 90% non-ionic surfactant at 0.25% v/v using a track sprayer calibrated to deliver 216 L ha^{-1} of spray solution. Plants were visually evaluated for necrosis 14 d after the

herbicide application. Plants surviving the herbicide application were scored as hybrid individuals.

All of the *B. rapa* var. *chinensis* receptor plants produced viable seed. Germination varied by receptor plant, ranging from 31 to 99% (Table A-1). None of the control plants survived the herbicide application. Five of the *B. rapa* receptor plants produced offspring that were resistant to the imazamox herbicide application indicating that hybridization between the *B. rapa* receptor plants and the herbicide resistant canola occurred. The greatest rates of hybridization (17%) occurred with the receptor plants that were located closest to the herbicide resistant canola field (Table A-1). However, herbicide resistant hybrid individuals were produced on receptor plants 194, 230, and 360 m away from the canola field (Table A-1). No herbicide resistant individuals were found in the offspring of receptor plants at a distance greater than 360 m from the herbicide resistant canola.

The results of this preliminary study demonstrate both that hybridization of these two species can occur under field conditions, and that distance from the pollen source influences the rate of hybridization. While no hybrids were found further than 360 m from the pollen source, pollen movement may be much greater. Our study only used 18 receptor plants, and was limited to a maximum distance of 729 m. Further studies should be conducted utilizing a larger number of receptor plants, and encompassing a greater area to validate the results of this preliminary study.

Table A-1. Receptor plant number, distance of each plant from the pollen source, total seeds produced on each receptor plant and percent germination of that seed, number of herbicide resistant individuals produced by each plant, and percent outcrossing from the crossing distance experiments conducted at the Hyslop research farm.

Plant Number	Distance from pollen source (m)	Total seed	Resistant individuals	Germination	Outcrossing
		---------- # ------------		------------ % -----------	
680	184	68	0	69	0
683	242	9	0	78	0
690	290	79	0	73	0
676	23	109	8	42	17
679	635	68	0	99	0
681	411	17	0	88	0
684	373	49	0	67	0
689	729	13	0	92	0
691	521	36	0	31	0
682	150	16	0	75	0
692	658	205	0	88	0
688	360	59	1	88	2
675	31	83	6	43	17
686	355	15	0	93	0
687	601	20	0	85	0
685	194	40	1	95	3
678	449	85	0	96	0
677	230	84	1	98	1

Figure A-1. Location of *B. rapa* var, *chinensis* inbred receptor plants (●), and the Imidazolinone resistant canola field (*X*) at Hyslop research farm in 2009.