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The Evolution of Natural Plant Communities through Crop Migration and Crop-to-weed Gene Flow

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THE EVOLUTION OF NATURAL PLANT COMMUNITIES THROUGH CROP
MIGRATION AND CROP-TO-WEED GENE FLOW

THE EVOLUTION OF NATURAL PLANT COMMUNITIES THROUGH CROP
MIGRATION AND CROP-TO-WEED GENE FLOW

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology

By

Meredith Schafer
University of Arkansas
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University of Arkansas

ABSTRACT

With the rising demand for food and biofuels, the efficiency of crop production has become vital to assuring food security. Genetically engineered (GE) crop varieties are broadly thought to be a solution for improving the efficiency of crop production, but concerns regarding their release have heightened as more discoveries have focused on the influence of genetically engineered crops on native and weedy plant species. Risk assessment of GE crop varieties focuses on two main areas: feralization of crop systems and crop-weed hybridization. Risks include naturalization, transgene introgression, and the formation of novel genotypes in natural systems. In this study, roadside surveys and sentinel plant experiments were conducted in North Dakota, U.S.A. in order to assess the risks facing natural plant communities by crop migration and crop to weed gene flow. We documented the establishment of transgenic, persisting canola (*Brassica napus* L.) populations on a large spatial scale in the U.S.A. and the development of novel genotypes within these populations. We also identify species and population level differences in hybridization rates between transgenic canola and weedy *Brassica* species within a commercial field environment.

This thesis is approved for recommendation
to the Graduate Council.

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DEDICATION

This thesis is dedicated to my fellow biology graduate students, my friends and family, and most importantly, my husband, Brett. Thank you for your encouragement, support, and love throughout this process. It has meant the world to me.

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INTRODUCTION

Humans are now facing a global challenge: how to meet the demand for food imposed by an increasing human population size. It is estimated that the world human population size will reach 9 billion by the middle of the 21st century (Godfray et al. 2010). Research shows that food production will need to double in order to bring food security to so many people and fulfill demand for biofuels (Foley et al. 2011), but with nearly 50% of the earth's arable land surface already managed in some form, the solution to this problem will be more complex than simply increasing cultivated acreage (Kareiva et al. 2007). Increased crop production has already led to large amounts of deforestation in the tropics causing a strain on ecosystem function, loss of soil carbon, and localized extinctions. Part of the complexity will be fulfilling the demand for food while remaining environmentally sustainable.

Solutions to the demand for food must focus on the efficiency of current agricultural practices. Goals should include increasing yield, maintaining harvest purity, decreasing amounts of herbicides and pesticides used, and the ease of crop management (Foley et al. 2011). The development of genetically engineered crop varieties has been credited with increased productivity over the last 20 years (Federoff and Cohen 1999, but see Gurian-Sherman 2009). In the United States alone, over 42.8 million ha of genetically modified crops are in production (Marvier and Van Acker 2005). Genetically modified varieties have been developed for cotton, soybean, canola (oilseed), carrot, potato, and corn (Snow and Palma 1997), but the release of GE crops has not proceeded without concern. Almost immediately after their release, questions of potential environmental impacts were raised. The risk assessment of GE crops primarily addresses concerns regarding the escape of GE transgenes into natural environments. The introduction of GE transgenes can be the result of GE crop species persisting outside of

cultivation and/or the movement of transgenes due to crop-weed hybridization allowing introgression of transgenes into native species. This risk of invasiveness is enhanced by the presence of GE transgenes that have the potential to increase the fitness of the feral crops in natural environments and increase the fitness of crop-weed hybrids allowing the introduction of novel genotypes into native populations (Wolfenbarger et al. 2000, Snow et al. 2003). In the case of *Raphanus sativus* L. (radish), hybrids with cultivated radish proved to have higher fitness than wild populations due to increased seed production (Klinger and Ellstrand 1994). With higher fitness, hybrid plants could reduce native plant population sizes and lead to the decline of native alleles.

The first step in the risk assessment of GE crop varieties is to examine their potential for persistence outside of cultivation. The isolation of crop varieties to cultivated fields is virtually impossible because seeds are easily lost during transport and processing, and volunteer plants within cultivated fields also provide a large seed stock for the transfer of seeds to undesired locations (Marvier and Palma 2005). Feral crop populations have been documented for canola, rice, wheat, sugar beat, and many others (Bagavathiannan and Van Acker 2008). Though reports of escaped domesticated plants have been made in many other countries, it has been largely overlooked in the U.S.A. In the U.S.A., reports of crop escapes are limited to a field study in Oregon on creeping bentgrass (*Agrostis stolonifera* L.) (Watrud et al. 2004). Feral crop populations have been shown to become weeds themselves in both cultivated and natural areas (DeWet and Harlan 1975). Their presence can decrease harvest purity and impact native plant populations through competition for resources. Novel traits possessed by many feral crop species can result in selective advantage over other species and provide the potential for the development of novel genotypes through hybridization.

The next step in the risk assessment of GE crop varieties is to quantify the amount of hybridization occurring between GE crops and sympatric weeds within their range of production. Almost all crop systems, specifically the top ten crop species in the world, have at least one sympatric, sexually compatible weedy species within their distribution (Ellstrand et al. 1999). Crop-weed hybridization allows for the development of novel genotypes and may disrupt natural plant populations. In some cases hybridization can lead to the extinction of a plant species and problems in crop management (Ellstrand 2003). Concerns of crop-weed hybridization were expressed by many biologists long before the development of genetically engineered crops, but now researchers can use these GE transgenes as genetic markers to track gene flow from cultivated species to their weedy relatives. The availability of GE markers can shed new light on the impacts of crop-weed hybrids on natural plant systems.

One such GE trait lies in the development of herbicide resistant crops. Herbicide-resistant crop varieties have been commercially released for soybean, canola, maize, and cotton (Owen and Zelaya 2005). The development of herbicide-resistant weeds through crop-weed hybridization has become a problem for crop management and the maintenance of harvest purity. It has been argued that increased production of herbicide-resistant crops will only increase our reliance on herbicides (Snow and Palma 1997). This has been shown in soybean systems due to the evolution of herbicide-resistant weeds. Herbicide-resistant weeds were first discovered just three years after the release of herbicide-resistant soybeans (Owen and Zelaya 2005). The evolution of herbicide-resistant weeds poses a threat to crop management and the preservation of biodiversity. Not only do these herbicide-resistant weeds have a potentially higher fitness than natural species, but they can infest cultivated fields leading to a decreased yield and contaminate the harvest. Herbicide-resistant weeds will have a competitive advantage over other species in

areas where herbicide is applied (Klinger and Ellstrand 1994). Their relative fitness to other species may be different in areas without herbicide application, but the persistence of herbicide-tolerant weeds has been documented for many systems. Over 174 weedy species have shown herbicide resistance (Owen and Zelaya 2005). These shifts in weed populations occur at a much faster pace than argued by many weed scientists and, until recently, many evolutionary ecologists (Ellstrand 2003).

To accurately assess the impacts of GE crops on native populations, feralization and crop-weed hybridization must be examined. This will be of increasing importance as crop varieties are being developed with multiple genetically engineered traits. The goal of this thesis is to examine the risks of transgene introgression into native plant populations imposed by the genetically engineered crop system *Brassica napus* L. (canola).

STUDY SYSTEM

Canola, *Brassica napus* L., is an ideal model system with which to examine the threats to native plant species imposed by genetically engineered crop varieties. “Canola” itself is a term registered by the Western Canadian Oilseed Crushers Association (“Canadian oil, low acid”). Canola was developed in the 1970s by Keith Downey and Baldur R. Stefansson to have a lower erucic acid level than its native counterpart, rapeseed oil (Berglund et al. 2007). Rapeseed was used as an industrial lubricant in WWII, but was not approved for human consumption until Downey and Stefansson developed “canola” with a lower acid level. It is now marketed as a vegetable oil with a low fat content (NYSE: ICE). The global production of canola/rapeseed averages 48 million metric tons annually. Canada and the U.S. are the largest producers and exporters of canola (Chance 2009).

Brassica napus L. is an allotetraploid produced through the hybridization of diploid *Brassica rapa* L. (wild mustard) and *Brassica oleracea* L. (cabbage). It has the potential to hybridize with at least 15 other *Brassica* species (Fitzjohn et al. 2007). These *Brassica* species include many that are noxious weeds in the U.S., specifically, *B. rapa* L. (wild mustard), *B. nigra* L. (black mustard), and *Sinapis arvensis* L. (field mustard). All three of these species have overlapping distributions with cultivated *B. napus* L. (USDA PLANTS database 2012).

CANOLA IN THE U.S.

The U.S. is the second largest producer of canola globally. In 2010, 1.45 million acres of canola were planted with an estimated value of \$471 million (USDA/NASS). Ninety-two percent of the canola in the U.S. is grown in North Dakota (Berglund et al. 2007). Within North Dakota, the largest areas of production are in the north-central and north-eastern portions of the state. Cavalier County, North Dakota, is the largest producer of canola in the state with over 200,000 acres planted each year (USDA/NASS). Over 90% of the canola grown in the North Dakota is genetically engineered for herbicide resistance (Ron Bernada, pers. comm.).

HERBICIDE-RESISTANT CANOLA AND TRANSGENE MOVEMENT

Canola, engineered for herbicide resistance, was commercially released in 1995. The most popular cultivars of herbicide resistant canola are biotech products engineered by the insertion of a gene that occurs naturally in soil-dwelling microbes. Glyphosate (RoundUp™) resistant and glufosinate (LibertyLink™) resistant canola are the two most common herbicide-resistant canola cultivars. A third type of herbicide resistant canola now available commercially is the result of mutagenesis rather than genetic transformation (Clearfield™) and will not be

addressed in this study. The presence of the protein 5-enolpyruvylshikimate-3-phosphate synthase, *CP4 EPSPS*, conveys resistance to glyphosate while the protein phosphinothricin-N-acetyl transferase, *PAT*, conveys resistance to glufosinate.

Canola is prone to seed shattering and produces seeds that are small in size but large in quantity. *Brassica napus* L. seeds average 1.8-2.7 mm in diameter (Warwick 2010). They are easily wind-dispersed and lost during transport. Seed shattering has also led to large amounts of volunteer canola plants in subsequent years of production. These volunteer populations serve as a large source for the movement of transgenes beyond the bounds of agricultural fields. These characteristics have led to the global reports of persistent, feral canola populations. Reports of feralization have been made in Canada (Beckie et al. 2003), Australia (Rieger et al. 2002), France (Pessel et al. 2001), Great Britain (Crawley and Brown 1995), Japan (Aono et al. 2006) and now Switzerland (N. Schoenenberger, pers. comm.). In Japan and Canada, the persistence of feral populations has led to the creation of novel genotypes with resistance to multiple herbicides, a trait not commercially produced (Aono et al. 2006, Knispel et al. 2010). These feral GE populations could be a reservoir of novel alleles in native plant populations. They also contribute to the increased weediness of *B. napus* itself. It is surprising that with worldwide reports of feral canola populations, to date there has not been a similar report made in the U.S. This must raise questions about the efficiency of monitoring efforts and protocols that are currently in place.

The ease with which canola hybridizes with its sexually compatible relatives also raises concerns. Although canola is self-compatible (able to self-fertilize), outcrossing rates are estimated to be 12 to 55% (Légère 2005). Vectors for pollen movement include both wind and insects. Bumblebees (Hymenoptera: *Bombus*) are the most frequently documented insect

pollinators of canola and have the ability to transfer pollen over long distances (Cresswell et al. 2002, Ramsay et al. 2003). Average foraging ranges for bees lie between 200 - 650 m (Hines and Hendrix, 2005; Wolf and Moritz, 2008).

In this study, examination of crop-weed hybridization will focus on three main *Brassica* species: *B. napus*, *B. nigra*, and *S. arvensis*. All three of these species are commonly found in North Dakota on roadsides, drainage ditches, disturbed areas, and cultivated fields. Their presence has been shown to decrease crop yield in some cases (Warwick 2010). Greenhouse and/or field experiments with these species have all shown the potential for hybridization (Légère 2005, Londo 2010). Rates of hybridization in greenhouse studies have proven very low for *B. nigra* and *S. arvensis* (Lègerè 2005).

Due to canola's relationship to *B. rapa*, it has been the most studied weedy species for transgene movement in this system. *B. rapa* is considered a noxious weed in the U.S. and a pest to crop management. Rates of hybridization between canola and *B. rapa* average from 0-36% in field experiments and 13.6% in commercial fields (Légère 2005). Herbicide-resistant *B. rapa* can persist in canola stands and decrease harvest purity. Examining the rate at which hybridization occurs between *B. napus* and *B. rapa* within areas of large commercial production in the U.S. will give insight into the effectiveness of monitoring protocols and management techniques.

Population-level variation in crop-weed hybridization has not been given as much attention. Identifying the most threatened populations to crop-weed hybridization will be important for conservation efforts. Winter varieties of herbicide-resistant canola are now being researched and produced in Alabama, Illinois, Kansas, Oklahoma, Texas, Montana, among others (Stamm and Dooley 2011). This could increase the range of canola production across the U.S., thus widening the areas of influence GE canola could have native plant species and

populations. It is possible that particular populations of weedy *Brassica* species are more susceptible to transgene movement than others. Understanding the most sensitive areas to transgene introgression will be vital to future conservation efforts of native alleles.

Therefore, the goals of this project were two-fold:

- 1) to assess the escape of canola (*B. napus*) on a large spatial scale in the U.S.; and,
- 2) to examine species and population level differences in the rate of crop-weed hybridization between canola and weedy *Brassica* species.

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THE ESTABLISHMENT OF GENETICALLY ENGINEERED CANOLA POPULATIONS IN THE U.S.A.

INTRODUCTION

Currently, more than half of the earth's land surface is managed in crop or forage species (Kareiva et al. 2007). The efficiency of our agricultural processes is of growing importance in order to meet the increased demand for food and new energy forms. Genetically modified crops have widely been considered a solution to the need for increased crop yield and efficiency. But the commercial release of genetically modified crops has not gone without concern. Concerns involve the escape of GM transgenes into native and weedy populations. Escape can be in the form of feral crops and crop-weed hybridization. The influence of transgene escape on natural species has been difficult to assess. The possible impacts of genetically modified varieties on native and weedy species are of particular interest. One potential impact is the introduction of novel traits into native populations. These novel traits could be beneficial or could lead to the reduction of natural populations and potentially the removal of native alleles. The confinement of GM transgenes has also proven to be a difficult task. Almost all crop systems, specifically the ten major crops in the world, have at least one weedy relative with whom they are sexually compatible allowing escape of transgenes (Ellstrand et al. 1999). Though many countries have reported feral crop species, in the United States, the only evidence of escape has been in a field study of creeping bentgrass (*Agrostis stolonifera*) where pollen migration was shown (Reichmann et al. 2006).

Canola (*Brassica napus* L. (Brassicaceae)), an oilseed crop, is a man-made hybrid created through the hybridization of *B. rapa* (wild mustard) and *B. oleraceae* (cabbage). It is sexually compatible with more than 15 of its weedy relatives (FitzJohn et al. 2007). Today, canola is

grown on more than 39 million hectares globally (FAOSTAT 2008). In 1995, canola that had been genetically modified for herbicide resistance was commercially released. Soon after its commercial release, transgenes from GM canola were found in plants growing outside of cultivation in Canada (Warwick et al. 2003). In the fifteen years since its release, GM canola varieties have reportedly escaped cultivation in Canada (Warwick et al. 2003), Great Britain (Wilkinson et al. 2003), Japan (Anono et al. 2006), France (Pessel et al. 2001), and Australia (Reiger et al. 2002). In the United States, GM canola was released for production in 1998 (Hall et al. 2000). North Dakota leads the nation in canola production with 92% of domestic production (Berglund et al. 2007). More than 90% of the canola planted in the state is genetically modified (R. Beneda, Pers. comm.) for resistance to either glyphosate or glufosinate. In 2010, roadside surveys were conducted to determine density and distribution of escaped transgenic canola populations across North Dakota and to designate areas of environmental risk to native populations. The goal of our study was to determine if canola has escaped domestication and to determine if the escaped populations possessed GE transgenes.

ROADSIDE METHODS

To evaluate the presence and abundance of feral canola, GE and non-GE, eleven east-west transects were conducted across highways of North Dakota, USA. These transects began on 4 June and ended 23 July 2010. Stops were made every 5 mi (8.04 km) along the roadside. At each stop a 1 X 50 m transect was established on one or both sides of the road depending on roadside conditions. GPS coordinates of each stop were recorded. A total of 5600 km were traversed with 63.1 km of roadside habitats surveyed (1.1% of the driving distance). Surveys were performed prior to the onset of flowers in cultivated canola fields. When canola was present

in a transect, all identifiable *B. napus* plants were counted and density was recorded. Then a single individual canola plant was chosen at random, photographed, collected, and pressed. Each collected *B. napus* was tested for resistance to glyphosate and/or gluphosinate. Resistance was determined through protein assay by use of Traitcheck® lateral flow test strips (SIDX Strategic Diagnostics, Inc. Newark, DE. USA). Presence of the CP4 EPSPS gene confers resistance to glyphosate (RoundUp™) while presence of the PAT gene confers resistance to gluphosinate (Liberty Link™). Leaf material measuring 6 mm in diameter was mixed with 0.05 mm distilled water to form the testing solution. Traitcheck® strips were then used to determine resistance according to the manufacturer's instructions. There is a third form of resistance, Clearfield™, for which we were not able to test. Results of the surveys were archived and are now located at the University of Arkansas.

RESULTS

We show that the escape and establishment of canola populations is extensive across the state of North Dakota (Figure 1.1). Canola was present at 46% (288/634) of the sampling locations. Eighty percent (230/288) of the canola expressed at least one transgene: 41% (117/288) were positive for only the CP4 EPSPS transgene (glyphosate resistance); 39% (112/288) were positive for only PAT transgene (gluphosinate resistance); and 0.7% (2/288) were positive for both transgenes, a phenotype not produced by seed companies (Table 1.1). The densities at these locations ranged from 0 to 30 plants m⁻² with an average of 0.3 plants m⁻². Though surveys were conducted before cultivated canola was in bloom, 86.8% of the collected roadside canola plants were sexually mature. Sexual maturity of these plants ranged from presence of a flower bud to fully mature siliques. This suggests that roadside populations have

been established and are not simply seeds being blown from the fields. An inverse distance weighted analysis was run (ArcMap 10, 2010) on the densities of canola populations, the areas of greatest risk to natural populations were shown to be in the Northeast, North central, and Southeastern portions of North Dakota (Figure 1.2). These areas are associated with areas of high canola production, intersections of major roadways, and areas of canola processing. There are also processing plants close to these areas. Seed spill from trucks on major roadways and intersections could be a potential source of seed input, but roadside canola was also present in remote locations where there is little to no canola production, intersections, or processing facilities suggesting other means of seed input.

DISCUSSION

The extent to which transgenic canola has escaped cultivation is extensive. Our survey is the first step in examining the effects of feral crop species on native populations. The fact that so few cases of feral GE crop species have been documented has led many to believe there is no threat to natural populations, but consumers and researchers alike continue to express their concern about food sustainability and long-term effects of GE crops. This study allowed us to evaluate the mechanisms involved in the expansion of canola populations from cultivated fields to roadside habitats and the risks to natural populations.

Large densities of canola plants were observed in areas of high traffic and production which leads us to hypothesize that seed transport was a large seed source. Similar results have been shown in Canada and France (Knispel 2010 and Pivard 2008). In France, seed spill was shown to supply 15% of seed input while the majority of seed input came from neighboring fields (35-40%) (Pivard et al. 2008). It is not surprising that the majority of seed input came from

neighboring fields. Canola seeds are small and can easily be blown around by the wind. Also, due to canola's recent domestication, it still possesses qualities of uncultivated plants such as seed shattering and seed dormancy (Gomez-Campo 1999, Gulden et al. 2003). These wild characteristics could explain why we found mixed GE (glyphosate and glufosinate) canola populations in 9 of the dense populations. Our observation that the roadside canola plants were more mature than cultivated canola plants gives evidence for establishment. The discovery of "stacked" (resistant to more than one herbicide) canola plants also suggests that these populations are persisting for multiple generations and these stacked plants are evidence to the fact that biotech varieties have hybridized. Because varieties resistant to multiple herbicides are not produced commercially, these stacked plants have arisen through other means. One possible explanation is that the stacked plant is the result of hybridization within an established roadside population through pollen movement between phenotypes. Hybridization could also have occurred within a cultivated field with the seed then transported to the roadside through various means.

The densities at which we observed these feral canola populations raises questions as to the importance of roadside habitats and biological diversity. Many argue that these GE plants have no competitive advantage over native populations. Herbicide application was heavy on many of the roadsides in North Dakota. This gives a competitive advantage to resistant plants and allows them to overtake areas once inhabited by native plants. Because our study was the first to discover escaped GE canola in the U.S. while in other countries discovery was made soon after release, questions must be raised about the efficiency of our monitoring efforts. We need to evaluate how effective our protocols are in preventing crop escape and proliferation, as well as, the introduction of novel alleles into native populations.

With the demand for food greater than ever and only increasing, we must take advantage of biotechnology and varietal development. But this cannot come at the expense of native species. More research is needed to understand how gene flow to native and weedy species will influence native populations. Our identification of areas of greatest risk to native plants provides focus areas for future studies and monitoring efforts of the influence of feral canola on natural populations.

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TABLES AND FIGURES

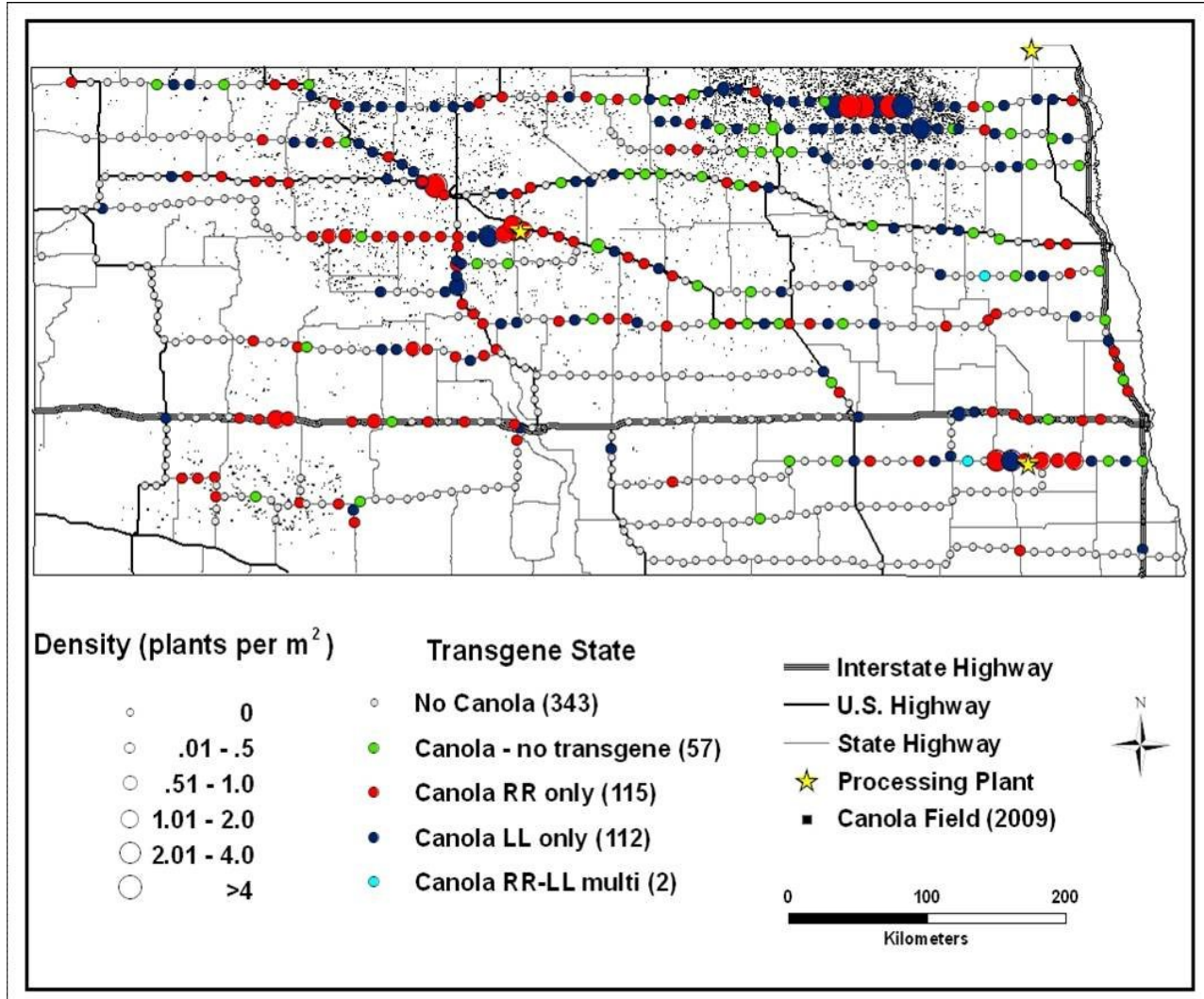


Figure 1.1. Distribution and density of feral canola populations in North Dakota road surveys (2010). Circles indicate locations of sampling sites; diameter of circle indicates plant density; gray circles indicate no canola present. The presence of genetically engineered protein in the vouchered specimen is shown by color: red – glyphosate resistance; blue – glufosinate resistance; yellow – dual resistance traits; green – nontransgenic. Canola fields are indicated by stippling based on 2009 USDA National Agricultural Statistics Service report (http://www.nass.usda.gov/Statistics_by_Subject/index.php?sector=CROPS). Stars show the locations of oilseed processing plants (3). Solid lines illustrate interstate, state and county highways.

	# of sites	Percent
Total Transects	634	
Canola Present	288	0.454
Transgenic	231	0.802
Liberty Link+ (LL+)	112	0.389
Roundup Ready+ (RuR+)	117	0.406
LL+ and RuR+	2	0.007
Non-transgenic		
Null	57	0.198

Table 1.1 Distribution of transgenic and non-transgenic canola in North Dakota transects.

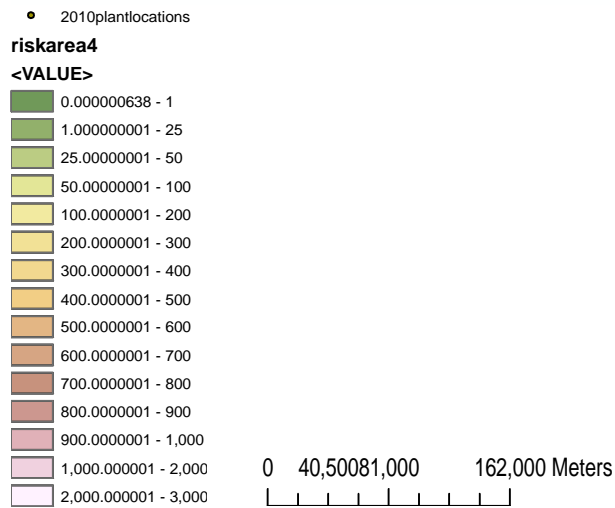
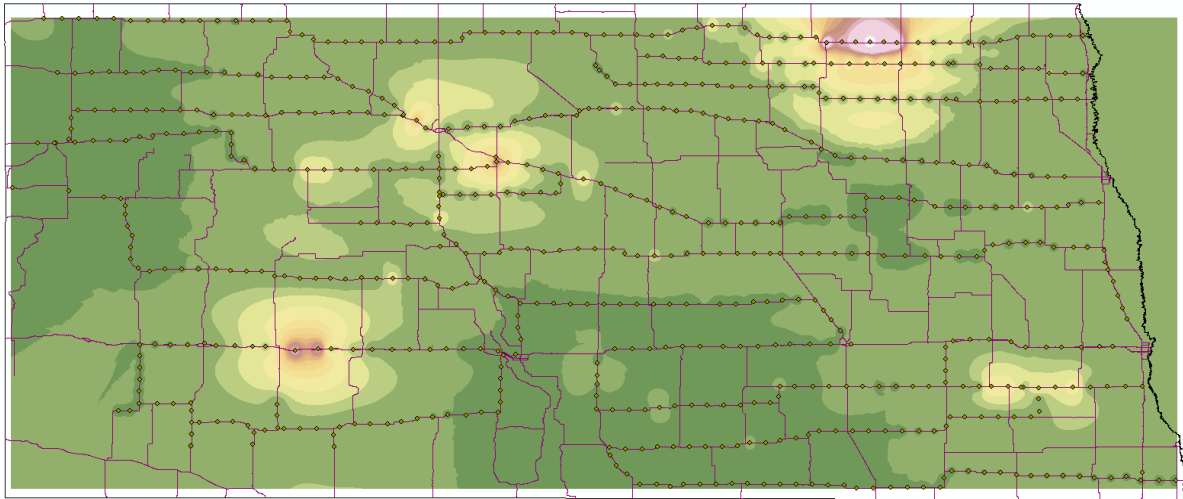


Figure 1.2. Areas of greatest risk to natural plant populations by feral GE canola. 2010. Circles represent survey locations on roadsides across North Dakota. Interpolation of the densities at these locations produces “hot spots” of environmental risk.

SPECIES- AND POPULATION-LEVEL VARIATION IN CROP-TO-WEED HYBRIDIZATION RATES FOR BRASSICACEOUS PLANTS

INTRODUCTION

Rising demand for food production has led to increased development of crop varieties aimed at increasing yield and maintaining harvest purity. Currently, nearly half of the earth's arable land surface is managed in crop or forage species (Millennium Ecosystem Assessment, Ecosystem and Human Well-being: Current State and Trends, Island Press, Washington, DC, 2005). The efficiency of our agricultural processes is important in meeting our demand for food (Foley et al. 2011). Genetically engineered (GE) crops have been pushed to the forefront as a solution to the need for increased yield and efficiency (Borlaug 2000). With the increasing production and use of GE crops, concerns have been mounting as to what influence they will have on natural systems. Environmental concerns about GE crops primarily lie with their impact on native and weedy species. The confinement of transgenes to cultivated fields and domesticated plants has proven to be a difficult task. All major crop systems, specifically the top ten crop species in the world, have at least one sexually compatible weedy species in their distribution (Ellstrand et al. 1999). The potential impacts of GE crops on native plant species include the introduction of novel genes into weedy populations and the reduction and potential extinction of native and weedy populations. Alternatively, the release of GE varieties has provided researchers with genetic markers to trace the transgene migration through crop-weed hybridization. This allows the investigation of monitoring efforts and protocols.

Many crop varieties have been developed for herbicide resistance. Herbicide resistance is, primarily, engineered with a gene that allows the crop to survive what would otherwise be a

lethal dose of herbicide. Resistance to herbicide is thought to provide a purer, higher yield and to allow easier crop management. Genes conveying herbicide resistance can be used as markers to trace outcrossing from GE crops to weedy species. Herbicide resistant crop varieties include soybean (*Glycine max* L.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L.), and many others (Légère 2005).

Canola, *B. napus*, is an ideal model system when examining potential impact on weedy species by GE crops. Ninety-two percent of the canola grown in the U.S. is grown in North Dakota (USDA NASS 2012), of which more than 90% is genetically engineered for herbicide resistance (Ron Bernada, pers. comm.). Canola genetically modified for herbicide resistance has been commercially available since 1995. It has been developed with resistance to the popular herbicides RoundUp™ (glyphosate) and Liberty Link™ (glufosinate). Though many GE crops are grown in areas where sexually compatible weeds are not found, canola is grown in areas with overlapping distributions with many sexually compatible relatives (Londo 2010). *Brassica rapa* L. (wild mustard), shows few barriers to outcrossing with *B. napus* (Jørgensen and Andersen 1994, Warwick et al. 2003, 2007), is considered a noxious weed in parts of the U.S. and a good study species for transgene movement. Other sexually compatible weeds include *B. nigra* L. (black mustard) and *Sinapis arvensis* L. (field mustard) (FitzJohn et al. 2007). These species are sensitive to hybridization and, therefore, are vulnerable to transgene movement. They are also obligate outcrossers meaning they cannot self-fertilize. These three species were chosen for this study due to their overlapping distributions with canola production areas in the U.S. and their ability to hybridize with canola as proven by greenhouse experiments (FitzJohn et al. 2007).

Though canola can self-fertilize, outcrossing rates range from 12 to 55% (Légère 2005). Pollen movement can occur from herbicide resistant canola to adjacent canola fields, GE or non-

GE, to volunteer canola plants in fields and roadside areas, and to weedy species in surrounding areas (Warwick et al. 2003, Légère 2005, Knispel et al. 2010). This pollen movement can result in issues of weed control, harvest purity, and even legal implication (Knispel 2008, 2010). In 2009 and 2010, field studies in Cavalier County, North Dakota were conducted to quantify the degree of hybridization occurring between canola and sexually compatible weeds. Sentinel plants were placed in commercial canola fields across Cavalier County, ND. The progeny of these individuals were then tested for resistance to quantify the amount of transgene movement occurring between canola and weedy sympatric species. The 2010 field study was designed to evaluate outcrossing differences at the population level by placing sentinel plants of differing *B.rapa* populations in commercial canola fields in North Dakota. We also assayed species and population level differences in the number of GE crop-weed hybrids produced between canola and the weedy *Brassicas*.

MATERIALS AND METHODS

In 2009 and 2010, individuals of sexually compatible weeds that have been documented to produce hybrids with canola were used as sentinel (non-resident) plants across ten commercial fields in Cavalier County, North Dakota, U.S.A. Ten individuals of each species or population were placed along a transect in each field. The progeny of these plants were then screened for herbicide resistance.

PLANT MATERIALS

Seeds used in the 2009 study were provided by colleagues from the U.S. EPA/NHEERL/WED in Corvallis Oregon. The seeds for our 2010 sentinel plants were collected

by several collaborators: Louisiana *B.rapa* seeds were harvested from plants collected by Cynthia Sagers at Rockefeller Wildlife Refuge, Sabine Parish, LA, USA (plant identity confirmed by Brent Baker-ANHC). The California *B. rapa* seeds were received from collaborator Steve Franks. Seed sources are from the central valley of California as described in Franks et al. (2008). The Oregon *B. rapa* seeds were collected by M. Bollman on the campus of the Oregon State University in Corvallis, OR, USA.

FIELD METHODS

Field studies were conducted in Cavalier County, North Dakota (Figure 2.1) in the summers of 2009 and 2010. The 2009 study consisted of sentinel plants (non-resident individuals) of three weedy Brassicaceous species: *B. rapa* (field mustard), *B. nigra* L. (black mustard), and *Sinapis arvensis* L. (wild mustard). The sentinel plants were started in Fargo, North Dakota at North Dakota State University in May 2009. *Brassica rapa* L. and *B. nigra* L. seedlings were transplanted in 4 L containers and kept in an enclosed outdoor area at North Dakota State University. Plants were regularly watered and tended through their vegetative stages. When the sentinel plants began to produce buds, one hundred individuals of each species were transferred from Fargo to Alsen, North Dakota in Cavalier County. Ten canola fields were chosen across Cavalier County as sites for sentinel plant placement. Before being placed in the field, plants were well watered and any open blooms were removed to ensure that only flowers pollinated in the field would be collected. In each field, one edge was chosen haphazardly and plants were placed along a transect running parallel to the field margin. Ten individuals of each species were placed in each of the ten fields by alternating by species along the transect. To reduce the amount of pollen transfer among individuals, plants were placed at 2 m intervals.

Plants remained in the fields for three weeks. While in the field, plants were watered regularly and any damaged plants were noted. The plants were placed in the fields on 7 July 2009 and removed on 28 July 2009. When potted plants were collected, any unopened buds were removed. Plants were allowed to mature in a greenhouse on the North Dakota State University campus until the seed siliques were well developed. Plants were then dried down and siliques harvested. Siliques were bagged with identification markers and paperwork identifying species and field placement. They were then shipped to the University of Arkansas for germination and herbicide screening.

On April 2010, seeds from three populations of *B. rapa* L.: Oregon, Louisiana, California, as well as, non-GE canola were germinated in 4 L containers. Methods followed from 2009. Plants were placed in the fields on 22 June 2010 and remained in the fields until 6 July 2010.

RESISTANCE SCREENING

Herbicide resistance screenings of seed progeny from the 2009 and 2010 sentinel plants were conducted at the University of Arkansas, Fayetteville. Herbicide screening began in Fall 2009 and ended in Fall 2011. Seeds were first harvested from siliques, then germinated in fifty-cell germination trays (Hummert Greenhouse Supply, St. Louis, MO, USA). Twenty-five seeds from each sentinel were selected haphazardly and planted in each half of the experimental tray. Each tray was then duplicated. One tray was used to test for RoundUp™ resistance while the other tray was used to test for Liberty Link™ (Ignite™) resistance. The trays were kept in a greenhouse with a temperature minimum of 15° C and maximum of 27° C. The trays were regularly watered. Before applying the herbicide, the number of tested seedlings was

documented. Once the seedlings reached the two to three leaf stage, 14-17 days after germination, they were sprayed with either RoundUp™ (glyphosate) or Liberty Link™ (glufosinate) herbicides at two times the field application rate (f.a.r.). For RoundUp™, a 19 ml/L solution was used. For Liberty Link™, a 20 ml/L solution was used. Herbicides were applied using 1L hand sprayers to ensure each of the seedlings was exposed to herbicide (Londo 2010). Trays were not watered the day after herbicide application to keep the solution from being washed away. Ten days after herbicide exposure, the number of resistant seedlings was determined. “Resistance” was confirmed through use of TraitChek™ immunological lateral flow test strips (Strategic Diagnostics, Inc., Newark, DE) or the presence of the CP4 EPSPS protein (which confers glyphosate herbicide tolerance) or the PAT protein (which confers glufosinate herbicide tolerance). To establish a positive, the seedling with the greatest damage yet still alive was tested first, then the next best-looking seedling was tested and so on, until a positive test was found or until all seedlings were confirmed negative (Londo 2010). The number of resistant seedlings was documented for each of the 2009 and 2010 sentinel plants. *S. arvensis* was not included in the herbicide screening process. Because *B. nigra* produced no herbicide resistant seeds and is more likely than *S. arvensis* to cross with canola (FitzJohn et al. 2007), *S. arvensis* was dropped from the screening protocol.

RESULTS

Outcrossing rates differed between canola and weedy *Brassica* species. There was no evidence of hybridization between canola and *B. nigra* (0/740) (Figure 2.2). In contrast, *B. rapa* had an overall outcrossing rate (resistant/tested) of 7.2% (283/3886). The outcrossing rates for *B. rapa* varied greatly across fields. The field maximum for *B. rapa* was 23.14% (100/432):

maximum for glyphosate resistance was 46.08% (100/217); and glufosinate resistance maximum was 32.11% (70/218). The maximum proportion of herbicide tolerant seeds produced by an individual *B. rapa* plant was 27.08% (13/48); for glyphosate resistance seeds, the maximum was 75% (18/25), and for glufosinate resistance seeds the maximum was 56.52% (13/23) (Table 2.1). At one of the ten fields, sentinel plants produced both glyphosate resistant seedlings and glufosinate resistant seedlings suggesting that either pollen type traveled from other canola fields.

Our 2010 study focused on variation in outcrossing rates among populations of *B. rapa*. Here we found evidence of population level differences in outcrossing rates. We detected significant differences in outcrossing rates among Oregon, Louisiana and California populations of *B. rapa*, and non-GE canola (Kruskal-Wallis test, Chi-square= 12.125 p-value= 0.0069). Significant outcrossing rates among *B. rapa* populations were also determined through a Kruskal-Wallis test ($P = 0.0033$), and were confirmed through pair-wise Wilcoxon signed rank tests. Outcrossing rates were highest for non-GE canola at 3.1% (50/1623). Louisiana *B. rapa* had an outcrossing rate of 1.99% (27/1351), Oregon *B. rapa* 0.50% (20/3926), and California *B. rapa* 0.33% (10/3026) (Table 2.1). Outcrossing rate varied greatly across the ten fields (G-test, $p < 0.0001$). The field range for Oregon was 0 to 5.6%, California 0 to 4.6%, Louisiana 0 to 10.8%, and non-GM canola 0 to 29.8% (Figure 2.2). Individual outcrossing rates also varied across populations: Oregon 0 to 17%, California 0 to 17%, Louisiana 0 to 26%. In addition, fields 1-4 produced progeny resistant to both glyphosate and glufosinate herbicides.

DISCUSSION

Outcrossing differences were discovered at the species and population level in this system. Genes conveying herbicide resistance were used as markers to identify hybridization between canola and sentinel plants placed in field margins. Herbicide resistance was detected in the progeny of these sentinel plants through exposure to herbicide and use of commercial test strips (Strategic Diagnostics, Inc. Newark, DE.). Identification of the most vulnerable species and populations to crop-weed hybridization plays a role in evaluating and developing monitoring efforts and conservation protocols.

The ability for canola (*B. napus*) to outcross to weedy species was not in question, but the rate at which it occurs under field conditions had not previously been measured in the U.S. Though our results show a large range in values, it is the extremes that are compelling. High rates of hybridization suggest that the escape of transgenes is a legitimate risk to the conservation of native plant populations. Our results show that monitoring efforts may be more efficient by focusing on certain species in the Brassicaceae family, specifically *B. rapa*. It has long been suspected that *B. rapa* would be the most sensitive weedy *Brassica* species to transgene movement due to its relation to canola. Canola itself is an allotetraploid ($2n = 38 = AACC$) produced through the combination of diploid *B. rapa* ($2n = 20 = AA$) and cabbage (*B. oleracea* L. $2n = 18 = CC$) (Londo et al. 2010). Canola easily hybridizes with *B. rapa*. Even though results showed zero outcrossing to *B. nigra* it should continue to be involved in future research on the influence of GE crops on weedy species. The *B. nigra* plants placed in the field produced a low seed set, and their progeny had a very low germination rate of 21% (740 seedlings out of 3500 seeds planted). This explains why five times the amount of *B. rapa* plants were tested for resistance versus *B. nigra*. Had more seeds been viable and available for screening, results could have been otherwise.

The timing of our 2010 field experiment may explain the lower rate of outcrossing seen in 2010 versus 2009. Our sentinel plants were germinated based on growing season dates from the previous year. However, large amounts of precipitation in 2010 delayed canola planting dates by a few weeks. Therefore, many of our experimental plants were flowering prior to the onset of blooms in the field.

Results of the 2010 sentinel plant study also suggest that certain populations of *B. rapa* were more susceptible to GE transgene movement than others. The population phenotypes varied greatly. Oregon was the most similar to canola in height, leaf size and shape, and inflorescence. Louisiana was similar to canola in height and inflorescence, but it had very large leaves creating resemblance to bok choy. California *B. rapa* was of reduced size and produced a smaller inflorescence than the other populations. Such phenotypic variation could explain the differences in outcrossing rate. Louisiana *B. rapa* proved to be the most susceptible to transgene movement. With the development of winter canola varieties now in progress, southern *B. rapa* populations could become exposed to gene flow from GE plants thus widening the threat of crop-weed hybridization beyond the bounds of North Dakota. Future research should examine whether the genetics of these different populations influences their susceptibility to transgene movement and identify patterns of genetic variation among populations. In addition, non-GE canola proved to have the highest rate of transgene movement than any of the *B. rapa* populations. This can lead to implications for growers of non-GE canola as GE transgenes could be unknowingly transferred to canola plants within their fields from neighboring GE canola fields. In some cases this has led to legal implications for farmers who did not purchase GE seed (Knispel 2008).

Due to the large variation in outcrossing rates across the fields, environmental factors influencing outcrossing rates should be further researched. The role of wind and bees in pollen

movement will be important to understand as vectors for transgene movement. Bumblebees specifically have been documented as the most frequent insect visitors to canola fields and are vital to the pollination of *B. rapa* as it is self-incompatible (Cresswell et al. 2002, Ramsay et al. 2003). Bee-mediated pollen movement allows transgene movement on a large scale. Though bee foraging ranges are variable, maximums for the genus lie between 800 m and 1750 m with averages between 200 m and 650 m (Wolf and Horitvz 2008, Hines and Hendrix 2005). Canola pollen in general has been shown to be viable as far as 1.5 to 2.5 km from its origin (Légère 2005). The ability of the pollen to move long distances poses a threat to native species and other canola plants outside of the field of origin. Threats consist of increased weediness in fields due to hybridization with native species, the transfer of GE transgenes into non-GE, organic and GE fields, and canola itself becoming a nuisance to crop management. Our discovery of glyphosate and glufosinate resistant progeny from the same field suggests pollen is moving from other fields or field margins into cultivated canola fields.

The efficiency of crop management protocols and agricultural practices will be of increasing importance as human population level increases. Genetically engineered crop varieties have long been considered the solution to our problem; but concerns regarding their release are becoming validated as research increases on GE crops influence on native and weedy species (Simard 2002, Légère 2005, Londo 2010). Not only are herbicide resistant “superweeds” of concern, but movement of GE pollen between separate agricultural fields is also of concern. Hybridization of these kinds can lead to increased weediness, increased herbicide costs, decline of harvest purity, and legal implications. We must also understand the necessity of conserving native plant communities. The importance of maintaining biological diversity cannot be overlooked as we look for solutions for food security (Foley et al. 2011). Continuation of

research on GE canola's influence through hybridization can serve as a model for other crop systems. This study is simply the first step in understanding the effects of GE crops on native species and populations.

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TABLES AND FIGURES

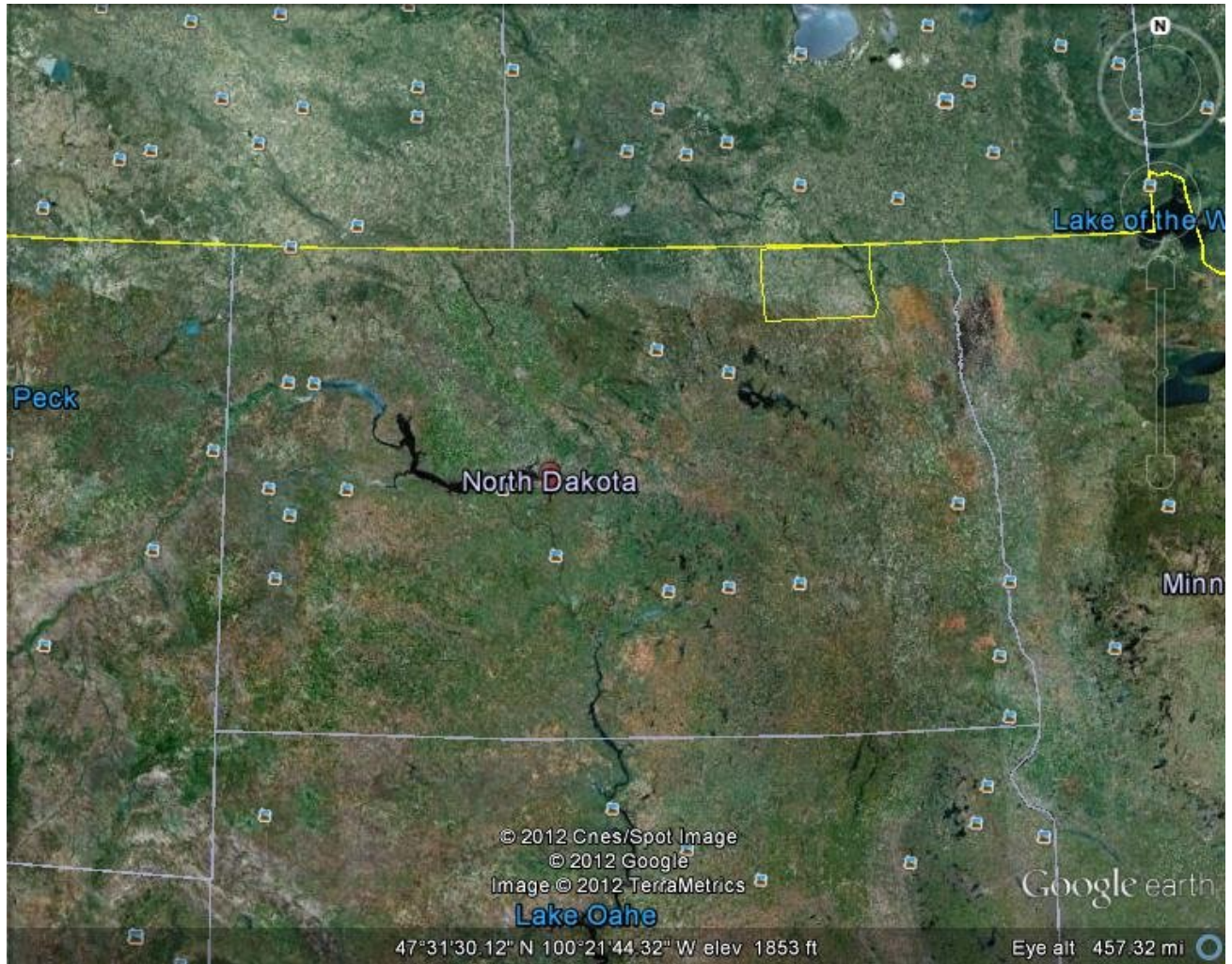


Figure 2.1. Map of Cavalier County, North Dakota. Location of the 2009 and 2010 sentinel plant studies. The yellow polygon outlines Cavalier County, ND in the northeast region of the state. This county is bordered by Canada on its north side.

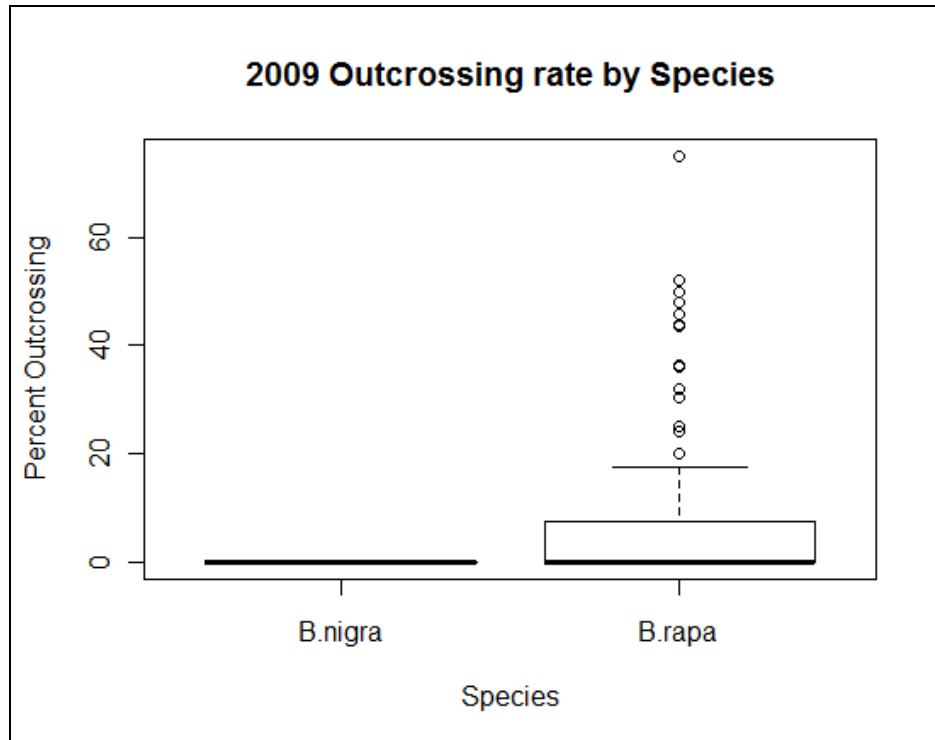


Figure 2.2. The 2009 outcrossing rates by species. *Brassica nigra* had a zero percent outcrossing rate. The outcrossing rate for *Brassica rapa* ranged from 0 to 56.52%.

A. 2009	Number resistant	Number tested	Percent
<i>Brassica nigra</i> L.	0	740	0.0
<i>Brassica rapa</i> L. (OR)	283	3886	7.20
Field Maximum	100	432	23.14
Glyphosate	100	217	46.08
Glufosinate	70	218	32.11
Individual Maximum	13	48	27.08
Glyphosate	18	25	75.00
Glufosinate	13	23	56.52
2010 Results			
Canola (<i>Brassica napus</i> L.)	50	1623	3.10
Field Maximum	9	136	6.60
Glyphosate	10	103	9.70
Glufosinate	20	67	29.80
Individual Maximum	10	33	30.30
Glyphosate	10	13	76.0
Glufosinate	19	24	79.0
B. <i>Brassica rapa</i> L.			
Oregon	20	3926	0.50
Field Maximum	12	432	2.77
Glyphosate	5	218	2.29
Glufosinate	12	212	5.66
Individual Maximum	3	39	7.69
Glyphosate	3	25	12.0
Glufosinate	3	17	17.64
Louisiana	27	1351	1.99
Field Maximum	25	426	5.68
Glyphosate	1	193	0.51
Glufosinate	24	222	10.81
Individual Maximum	6	48	12.50
Glyphosate	1	19	5.68
Glufosinate	6	23	26.08
California	10	3026	0.33
Field Maximum	6	249	2.40
Glyphosate	0	25	0
Glufosinate	6	130	4.61
Individual Maximum	0	25	0
Glyphosate	0	25	0
Glufosinate	4	23	17.39

Table 2.1. The results of the 2009 field study. A. outcrossing rates between species. B. Ranges of outcrossing rates within *Brassica rapa* L.

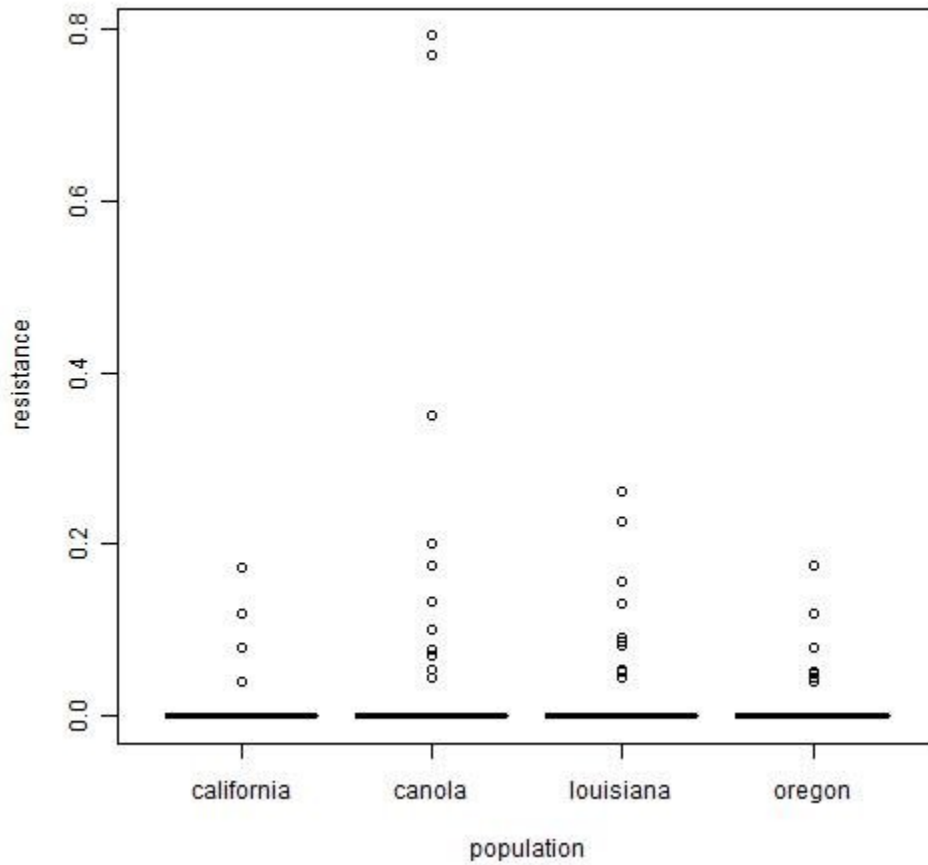


Figure 2.4. The 2010 outcrossing rates for three populations of *B. rapa* and non-GE canola. The mean outcrossing rates are shown for each population/species with standard deviations.

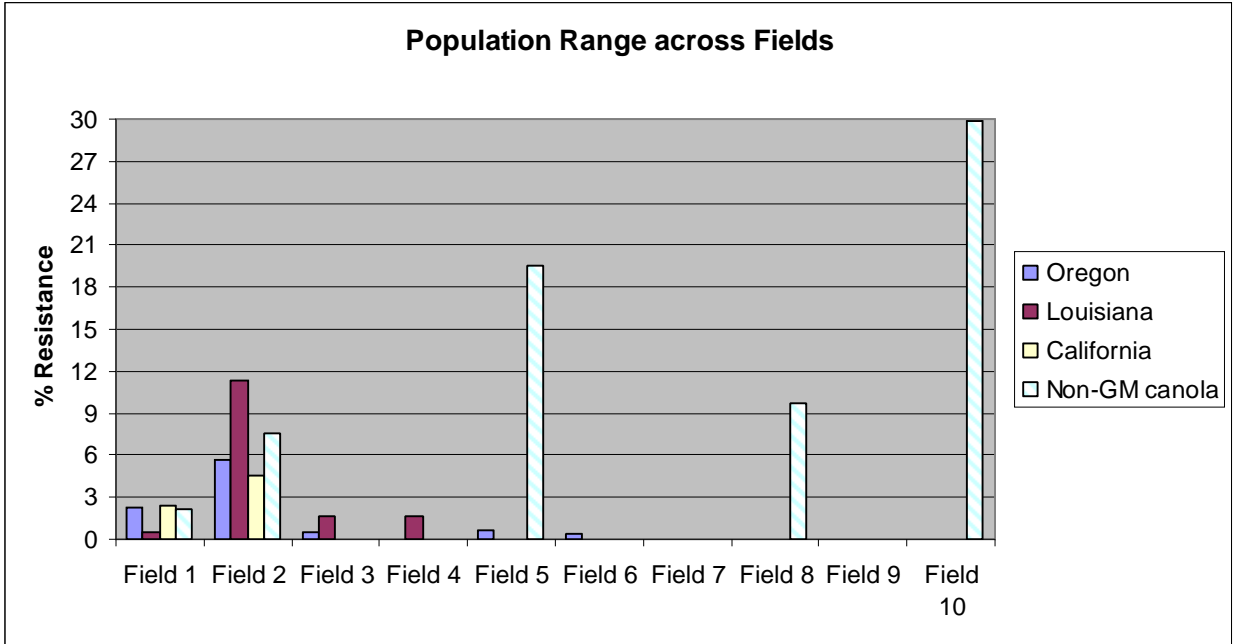


Figure 2.5. 2010 Incidence of transgenic seeds by field. Variation in percent of transgenic progeny documented for each population across 10 experimental fields.

CONCLUSION

In the U.S., the risk of genetically engineered transgenes entering natural plant systems is very real. This study documents the escape of the commercial crop, canola (*Brassica napus* L.), on a large scale in the U.S. We also observed canola's ability to produce novel genotypes outside of cultivation, and GE canola's rate of hybridization with sympatric weeds and non-GE canola under field conditions. Tracking the environmental impacts of biotech products will be of utmost importance with the increased development and production of genetically engineered varieties and varieties containing multiple transgenes, yet the lapse in time between our results and similar reports in Canada, Australia, France, Germany, the U.K. and Switzerland, raises questions about monitoring protocols and efforts in the U.S. (Aono et al. 2006, Beckie et al. 2003, Crawley and Brown 1995, Pessel et al. 2001).

The lapse in time to detection may be evidence of a lack of environmental risk in transgene escape and suggests that these plants do not have selective advantage or increased fitness. However, in areas of herbicide application, like roadsides and field margins, herbicide-resistant plants will be selected for and will persist. Similar reports were made for *Raphanus sativus* L. (radish) (Klinger and Ellstrand 1994) and *Helianthus annuus* L. (sunflower) (Snow et al. 1998). In these studies, GE hybrids proved to have a higher fitness than their wild counterpart. The introduction of transgenic plants into a natural ecosystem could have repercussions in terms of biodiversity and may threaten the conservation of roadside habitats which are vital in highly modified areas. These persistent feral canola populations could potentially disrupt roadside habitats, areas important for the conservation of many bee, bird, amphibian, and butterfly species (Ries et al. 2001, Hopwood 2008). This is especially true in highly fragmented areas where roadside grasslands and native plant species support these and other organisms. There should be

continued research on and monitoring of feral canola populations in North Dakota. Surveys should continue in order to understand the long-term effects of the presence of these feral populations on native roadside plant populations. Pinpointing the sources of seed input, examining pollen movement and hybridization between feral plants are the next steps in understanding the impacts of these populations. Some future directions for this study include stable isotope analysis of roadside canola. Stable isotope discrimination can be used as an estimate for water use efficiency and photosynthetic rate. Understanding where feral canola populations are most successful and the factors influencing their success will be important information for control of escaped populations.

Canola itself is becoming increasingly weedy within and outside of cultivated areas. Due to seed shattering and seed loss during harvest, canola produces weedy volunteers. In Canada, volunteer canola plants were found in 90% of surveyed fields (Simard et al. 2002). In North Dakota, volunteer canola densities rival that of cultivated canola (*C. Sagers per. obs.*). Because it is recommended that canola be grown on rotation, usually with winter wheat, herbicide resistant canola volunteers pose problems for farmers by increasing management costs and decreasing harvest purity. Hybridization of GE and non-GE canola will only increase the problems posed by weedy canola for farmers. Weedy GE canola and hybridization between GE canola and non-GE canola could also have legal implications for organic farming operations. Herbicide costs will also increase with the persistence of weedy canola; and the threat of canola resistant to multiple herbicides poses more problems for crop production and management in the future.

Hybridization between GE crops and weedy species within their distribution also raises concerns about the impact of GE crops on natural plant populations. This study shows that certain *Brassica* species have a higher risk of transgene introgression from GE canola than

others. Though we only observed outcrossing to *B. rapa* L., the potential for outcrossing to other weedy species is still a possibility. Our results in 2009 on the hybridization rate between GE canola and *B. rapa* L. (overall 7.2%, maximum for field 46.08%) are consistent with similar reports in Canada (Légère 2005). The large variation we discovered across fields leads one to believe that environmental and pollinator variation across fields plays a role in transgene movement. More research is needed on the role of bee and wind pollination in the movement of transgenes from GE crop to their weedy relatives. Future monitoring of the persistence of canola-weed hybrids and their fitness will be the only way to confirm whether transgene introgression is actually occurring and its effect on native plant systems. Perhaps the most interesting result from the 2010 sentinel plant study is the discovery of population level differences in hybridization rates. Understanding how the genetic variation within populations influences their susceptibility to transgene movement will be important for future monitoring of herbicide-resistant weeds in the U.S. Now that winter varieties of GE canola are being produced, areas of risk for transgene introgression into native plant populations are only increasing.

We must remember that in meeting the increasing global demand for food, we cannot compromise environmental sustainability (Millenium Ecosystem Report 2005). Solutions should also focus on other aspects of production efficiency. The processes involved in food transport could definitely be improved and could decrease the amount of food wasted between production and consumer. Thirty to 40% of harvested food is lost in transport in developed and underdeveloped countries (Godfray et al. 2010, Foley et al. 2011). While it may not be reasonable to eliminate the use of genetically engineered crop varieties, more research needs to be conducted to understand the long-term effects of transgene release.

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