2008

Measuring the Effectiveness of Isolation of Brassica napus L. Accessions During Caged Germplasm Regeneration

Von Mark V. Cruz
International Service for the Acquisition of Agri-biotech Applications

Charlie L. Rife
Blue Sun Biodiesel

John D. Nason
Iowa State University, jnason@iastate.edu

E. Charles Brummer
University of Georgia

Candice A. Gardner
United States Department of Agriculture

Follow this and additional works at: https://lib.dr.iastate.edu/eeob_ag_pubs

Part of the Ecology and Evolutionary Biology Commons, and the Genetics Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/eeob_ag_pubs/77. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Ecology, Evolution and Organismal Biology at Iowa State University Digital Repository. It has been accepted for inclusion in Ecology, Evolution and Organismal Biology Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Measuring the Effectiveness of Isolation of Brassica napus L. Accessions During Caged Germplasm Regeneration

Abstract
Gene flow, which is the successful movement of genes among populations by mating or migration of seeds or other propagules, has gained much interest in agriculture in recent years because of the wide-scale adoption of transgenic crops and concerns over transgene escape into the wild (James 2004; Messeguer 2003; Stewart et al. 2003). Brassica napus (rapeseed), together with maize and sugar beet, have been identified among the species for which cross-pollination and transgene escape are concerns (Treu and Emberlin 2000).

Disciplines
Ecology and Evolutionary Biology | Genetics

Comments
This article is from PGR Newsletter issue no. 154 (2008): 14.

Rights
Works produced by employees of the U.S. Government as part of their official duties are not copyrighted within the U.S. The content of this document is not copyrighted.
Introduction

Gene flow, which is the successful movement of genes among populations by mating or migration of seeds or other propagules, has gained much interest in agriculture in recent years because of the widespread adoption of transgenic crops and concerns over transgene escape into the wild (James 2004; Messeguer 2003; Stewart et al. 2003). *Brassica napus* (rapeseed), together with maize and sugar beet, have been identified among the species for which cross-pollination and transgene escape are concerns (Treu and Embertin 2000).

In the conservation of plant genetic resources, pollination-control methods to preserve the genetic integrity of regenerations include pollination by hand and the use of screen or fabric cages with insect pollinators. Failure of these methods to control contamination by any pollen source, regardless of whether the pollen is transgenic or non-transgenic, can compromise the integrity of the collections, which limits their utility to researchers.

Screened cages are widely used to enclose growing plants during germplasm regeneration, as in the case of *Capsicum* in New Mexico (Bosland 1993), and ornamentals, *Cucumis, Helianthus*, and *Brassica* at the North Central Regional Plant Introduction Station (NCRPIS) (NCRPIS 2002). The purpose of the cages is to maintain the genetic integrity of each accession by preventing cross contamination among adjacent accessions in the field while allowing enclosed plants to inter-mate with the help of insect pollinators. The NCRPIS cage system has been found effective in maintaining accession germplasm identity in *Helianthus annuus* and *Cucumis sativus* (Widrtechner et al. 1992; Wilson 1989), but no similar study has been conducted on this system for *Brassica*.

*Brassica napus* has a mixed mating system, where cross-
pollination occurs at an average of 30% (Warwick and Miki 2004). The rate of outcrossing can vary among populations and locations, ranging from 5% to 47% (Becker et al. 1992; Lavigne et al. 1998; Rakow and Woods 1987). Cross-pollination was thought to be effected only by insects in B. napus. However, Timmons et al. (1995) and Wilkinson et al. (2003) indicated that wind plays a significant role in its pollination, over even long distances. Substantial amounts of Brassica pollen are released into the air during flowering and can remain airborne (McCartney and Lacey 1991). Lewis (1983) and Sarkissian and Harder (2001) have reported that the diameter of Brassica pollen ranges from 24 to 27 μm. However, popular screens used for caging have a low resistance to wind (Bell and Baker 2000) and mesh openings of 280 to more than 1000 μm (BioQuip 2005). Considering the small size of B. napus pollen relative to the mesh holes of the cage screens, it is conceivable that pollen could pass through screens and effect cross-pollination. If pollen can pass through the screens, the probability of cross-pollination may be inversely proportional to distance between cages during regeneration. In addition, higher outcrossing rates could result for self-incompatible Brassica accessions (Hansen et al. 2003).

The potential for contamination is a concern in maintaining the integrity of accessions, especially those of outcrossing species during germplasm regeneration in genebanks (Ramanatha Rao and Hodgkin 2002). Because the regenerated plants often represent a subsample of the accession, the impact of gene flow events can be magnified. With small population sizes, gene-flow events can occur at higher rates with stronger effects (Goodell et al. 1997). This study was designed to determine if B. napus pollen contamination occurs under the standard cage regeneration protocols currently employed at NCRPIS when using Lumite® screening, and to determine the rate and significance of gene flow events during regeneration.

Materials and methods

Plant materials

Two varieties of B. napus with overlapping flowering times were selected: a conventional hybrid (Hyola 401), and its Roundup-Ready® (RR) isolate (Hyola 357RR), kindly provided by the Interstate Seed Company, West Fargo, ND, USA. Glyphosate-herbicide resistance provided a dominant-trait model for which a bioassay can be easily conducted.

Upon receipt, 200 seeds from each variety were germinated in the greenhouse and tested for RR-trait purity following a screening procedure modified from Reboud (2003). Glyphosate herbicide was applied at 1080 g/ha at 300 L/ha during the two-leaf stage and again two weeks later.
Field layout and planting

Two replications, each containing nine plots, were established on the Bennett Farm (41°59'36"N, 93°41'22"W) of Iowa State University (ISU) (Figure 1a). In each group, the centre plot was planted with Hyola 401 and the surrounding plots with Hyola 357RR. The design maximized the opportunity for the non-RR centre plots to capture pollen from an RR source given any possible wind direction. Direct seeding was done by using a V-belt push planter (Allan Machine Company, Nevada, IA, USA) following the row and plant distances of the NCRPIS protocol for Brassica planting (NCRPIS 2002). Non-RR plots were sown first to prevent possible contamination of the non-RR plots with RR seeds, and the planter was thorougly cleaned prior to planting each variety. The plots were thinned to 100 plants in 2 rows (50 plants per 5.5 m row) to give a density of 18 plants/m². To verify that no admixing of seeds occurred during planting, all the plants in the non-RR plots were checked for the presence of RR plants before flowering. Lateral-flow membrane strips (QuickStix™ Kit for Roundup Ready® Canola Leaf and Seed, Enviroylogix, Portland, ME) (one strip per two plants) were used to detect the expression of CP4 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) protein in leaf tissues and identify transgenic plants.

Screen cages, insect pollinators and plot care

The plots were covered with screen cages, each 1.5 m tall × 1.5 m wide × 6.0 m long before the plants began flowering (Figure 1b). The screen cages used at the NCRPIS were previously described by Widrlechner et al. (1996). The screen cages were spaced 4.5 m apart in the field. The Lumite® screen material of the cages has a mesh size of 7 × 6 per cm. The average size of the screen mesh hole is 1394 ±49 (s.e.) μm.

A colony of honeybees (Apis mellifera) in a 'nuc box' (16.8 cm × 50.8 cm × 27 cm) was placed in each of the non-RR cages when the first flower appeared and contained inside until the end of flowering. No pollinator insects were placed in the RR cages to ensure that pollen escape from the RR cages would be due to wind action and not due to escaped bees. No feral bees were observed during the flowering period.

Field fertility levels were high and no fertilizer was applied. No aphids or thrips were observed inside the cages during the season. Acephate insecticide was sprayed once during the plants juvenile stage to control alfalfa loopers (Autographa sp.).

Pollen and wind measurements

The direction and speed of wind during the flowering period were recorded at 10-minute intervals by a portable weather station.
placed in the centre of the field. Simple pollen traps were constructed following the method of Hoekstra (1965). The pollen traps have two circular sticky surfaces, each with an area of 38.5 mm². The static traps were positioned in four locations in the field (2.0 m from each cage) at the start of flowering (Figure 1a).

Pollen grain sizes were determined from flowers harvested from the RR and non-RR plants. Measurements were conducted using a Z2™ Particle Count and Size Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA) with a 100 μm aperture. Size measurements were obtained from 1.0 ml of pollen collected in Isoton® II Diluent (Beckman Coulter, Inc., Fullerton, CA, USA).

Detecting gene flow

Seeds were harvested from the cages of RR and non-RR varieties while the screens are still in place. Seeds from the non-RR cages were sown in the greenhouse in plastic nursery flats (543 x 279 x 62 mm (LWH); T.O. Plastics Inc., Minneapolis, MN, USA) with Fafard® Canadian Growing Mix no. 2 (Conrad Fafard, Inc., Agawam, MA, USA). The frequency of detection of RR progeny from the cages of non-RR plants provided an estimate of the rate of gene flow between accessions resulting from pollen escape and successful pollination. Out of the 9500 seeds planted, 9220 seedlings reached the two-leaf stage and were screened for resistance. Glyphosate herbicide was applied at 1080 g/ha in 300 L/ha during the two-leaf stage and re-sprayed after two weeks. Seeds germinated from both the original samples of Hyola 401 and Hyola 357RR served as susceptible and resistant controls. Contamination frequency (gene flow) was estimated by calculating the proportion of resistant seedlings, 14 days post-herbicide application. The results were validated by testing the surviving plants for expression of CP4 EPSPS protein by using lateral-flow membrane strips.

Results and discussion

Flowering began 45 days after planting and continued for more than three weeks. The RR plants started flowering four days earlier than the non-RR plants. The mean pollen size of RR plants was determined to be 29.20 ±0.07 (s.e.) μm and the non-RR plants 29.92 ±0.13 (s.e.) μm. These sizes are larger than those reported by Lewis (1983) or observed by Sarkissian and Harder (2001) in B. rapa. There was no significant differences between the pollen sizes of the non-RR and RR varieties (P<0.18).

Clumps of B. napus pollen (two or more grains) were detected in sticky traps outside cages after the initiation of flowering. The highest frequency of pollen detection was observed during the second week of monitoring, when the plants were at 50% flowering (Figure 2). There was no significant correlation between
the quantity of pollen trapped versus wind speed \((r=0.20; t_{0.05}^{23}=0.98, t_{	ext{crit}}=1.71)\), wind gust \((r=0.22; t_{0.05}^{23}=1.08, t_{	ext{crit}}=1.71)\) or wind direction \((r=-0.06, t_{0.05}^{23}=0.29, t_{	ext{crit}}=1.71)\). Aggregation, or clumping, is due to pollen stickiness and has been previously reported; it is believed to be an adaptation to favour dispersal by animals (Cresswell et al. 2004).

Mature pods were harvested 83 days after planting. Seed set on caged plants of the RR variety in the absence of insect pollinators was less than seed set from caged non-RR plants with bee pollinators, but not significantly different \((P<0.33)\). The results agree with Free and Nuttall (1968) and Lerin (1982), who found that there was comparable seed set between caged plants with pollinators and those without pollinators in *B. napus*. Pollinator insect activity may be more important in other *Brassica* species (e.g. *B. rapa*) with higher expression of self incompatibility (Thomas 2003).

At present, wind is believed to be a major initiator of cross- or self-pollination in *B. napus*, and bees are unlikely to have substantial effects on yield (Lerin 1982; Thomas 2003). Experiments conducted on *Brassica* grown in the still air of a glasshouse resulted in poor yields (Williams 1978). In our experiment, seed set occurred due to either self or sib fertilization. Without pollinators, this could have been effected by the wind, enabling the transfer of viable pollen to receptive stigmata due to shaking of the plants (Williams 1978). Results of wind tunnel experiments on *B. napus* flowers do not reject the possibility that stigmata can capture airborne pollen (Cresswell et al. 2004).

*B. napus* is an allotetraploid \((4x, 2n=38)\) and all currently commercialized transgenic canola varieties of this species carry two dominant genes for Roundup resistance, a copy each of the *cp4* EPSPS gene from *Agrobacterium* sp. and a modified glyphosate oxidoreductase gene (*goxv247*) based on *gox* from *Ochrobactrum anthropi* (Warwick and Miki 2004). Given the two copies of the resistance genes, the genotype for the tetraploid hybrid can be noted as RRxx (x denoting the null allele). The gametic output for an allotetraploid is expected to follow that of a diploid (Comai 2005). In *Brassica*, chromosome pairing was reported to be genetically controlled (similar to the *Ph1* mechanism in wheat) and varies among plant varieties (Jenczewski et al. 2003). In the complete absence of preferential chromosome pairing, the pollen \((n=2x)\) from the transgenic line would have three possible genotypes: RR, Rx and xx, occurring with probabilities of 0.25, 0.50 and 0.25, respectively. Due to these possible genotypes and associated probabilities, there remains a small amount of undetectable pollen flow. The proportion of pollen that has the null genotype cannot be determined empirically following our experimental design and procedure.
Two large seed samples from the non-RR harvest were planted in the greenhouse, resulting in 9220 seedlings (rep1=4523; rep2=4697), which were sprayed with glyphosate to test for resistance. A single plant in replicate 1 tested positive for herbicide resistance, and the subsequent lateral-flow strip test verified the presence of the transgene. These results correspond to a contamination rate of 0.01% (1:9220). If one accounts for bias created by undetected gene flow from the xx pollen, this estimate would only increase by 0.003% at most, or equal 0.013%. This estimate is within the genetic purity limit established by the Association of Official Seed Certifying Agencies (AOSCA), which allows a maximum of 0.25% contamination in certified canola seedlots and 0.05% for breeder and foundation seedlots (Friesen et al. 2003).

The contamination we recorded in the cage experiment is comparable to the hybridization rate observed by Scheffler et al. (1995) in B. napus test plots planted 200 m apart in an open field. It is also much lower than the observed contamination in small plots planted 1.5 m apart without cages (Funk et al. 2006). A zero percent geneflow between accessions is desirable, but difficult to attain in a cross-fertilizing crop such as Brassica. Insect exclusion cages, with coarse- or fine-weave netting, were reported by Ramsay et al. (2003) to reduce pollination in their study, but did not completely prevent it. We are confident that given the observed level of detected outcrossing (<0.02%), the current screened-cage production system utilized at the NCRPIS sufficiently isolates each accession to preclude contamination due to inadvertent pollen flow during regeneration.

Conclusion

This study demonstrated that pollen from a B. napus variety grown in screened cages in the field during germplasm regeneration can pass through the screen mesh currently in use by the NCRPIS and cause contamination across cages at a low frequency. The contamination occurred below the current AOSCA limit set for maintaining genetic identity, giving us confidence that the current method of regenerating B. napus in screen cages is functional. It is yet to be determined whether switching to a finer screen or increasing the distance between cages could completely eliminate pollen flow, and if levels of contamination vary across the more than 20 species of Brassica being conserved at the NCRPIS.

Acknowledgements

This is a journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 1018, and was supported by Hatch Act and the State of Iowa, and by............
the U.S. Department of Agriculture (USDA), Agricultural Research Service, Plant Introduction Research Unit. We wish to thank Mr Jim Johnson (Interstate Seed Co) for providing the canola seeds, Mr Mike Stahr (ISU Seed Science Center) for the test strips, Dr Madan Bhattacharyya, Dr Mark Westgate, Ms Maria Hartt-Eckerman (ISU Agronomy Department), and Dr Barbara Bingaman (NCRPIS, ISU Agronomy Department) for technical assistance and the use of laboratory and field equipment, and Mr Larry Lockhart, Mr Lloyd Crim and Mr Brian Buzzell (NCRPIS, ISU Agronomy Department) for help in land preparation, maintenance and cage assembly. We are also grateful to Dr Mark Widrlechner for reviewing the manuscript, and to the Fulbright Program of the U.S. Department of State’s support of VMVC during this study. Mention of commercial brand names in this paper does not constitute an endorsement of any product by the U.S. Department of Agriculture or cooperating agencies.

References


Treu R, Emberlin J. 2000. Pollen dispersal in the crops maize (Zea mays), oil seed rape (Brassica napus ssp. oleifera), potatoes (Solanum tuberosum), sugar beet (Beta vulgaris ssp. vulgaris) and wheat (Triticum aestivum). A Report to the Soil Association from the National Pollen Research Unit, University College Worcester, UK.


Williams I. 1978. The pollination requirements of swede rape.