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Benefits of Transgenic Insect Resistance in *Brassica* Hybrids under Selection

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Academic Editor: Peter Langridge

Abstract: Field trials of transgenic crops may result in unintentional transgene flow to compatible crop, native, and weedy species. Hybridization outside crop fields may create novel forms with potential negative outcomes for wild and weedy plant populations. We report here the outcome of large outdoor mesocosm studies with canola (*Brassica napus*), transgenic canola, a sexually compatible weed *B. rapa*, and their hybrids. *Brassica rapa* was hybridized with canola and canola carrying a transgene for herbivore resistance (*Bt Cry1Ac*) and grown in outdoor mesocosms under varying conditions of competition and insect herbivory. Treatment effects differed significantly among genotypes. Hybrids were larger than all other genotypes, and produced more seeds than the *B. rapa* parent. Under conditions of heavy herbivory, plants carrying the transgenic resistance were larger and produced more seeds than non-transgenic plants. Pollen derived gene flow from transgenic canola to *B. rapa* varied between years (5%–22%) and was not significantly

impacted by herbivory. These results confirm that canola-weed hybrids benefit from transgenic resistance and are aggressive competitors with congeneric crops and ruderals. Because some crop and crop-weed hybrids may be competitively superior, escapees may alter the composition and ecological functions of plant communities near transgenic crop fields.

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1. Introduction

Hybridization is an essential tool in the traditional development of new crop varieties. Hybrids frequently express novel traits or combination of traits, which allows selection of new varieties more suited to the local environment. Similarly, hybridization in nature followed by natural selection produces genotypes more closely adapted to local conditions [1]. As such, hybridization is now understood as an important mechanism of plant evolution and diversification [2]. In agricultural systems, a number of important weeds have evolved from hybrids of wild and domestic species [3,4]. The study of crop-wild, and crop-weed hybrids has gained renewed interest following the introduction of biotech crops. Since then, non-transgenic crops have been found to carry genes engineered for beneficial traits, which arose through hybridization among cultivars. Examples include cotton, creeping bentgrass, canola, alfalfa, corn, soybean, and sugar beet (reviewed in [5]). On occasion, transgenic crop-crop or crop-wild hybrids have been found following small-scale field trials of genetically modified cultivars [6]. Despite the importance of hybridization for the evolution of new weed species [4], the ecologies of crop-weed and crop-wild hybrids have received limited study outside of agricultural fields. This project was undertaken to investigate the ecologies of hybrids of domesticated and feral *Brassica* species, including hybrids carrying the pesticide gene *Bt Cry1Ac*, in a simulated natural environment subjected to competition and herbivory.

A chief concern of crop-weed hybridization is the uncertain ecologies of transgenic hybrids in the native landscape [7]. The frequency of transgenic forms will depend upon continued migration of alleles from cultivated plants, the benefits of the transgene under selection, and the costs of the transgene when no selective pressure is present [8]. Costs may be due to pleiotropic effects of the transgene, an increase in gene dosage, or mutational changes induced by plant transformation [9]. If costs are high and beneficial selection rare, fitness of the hybrid will be reduced relative to non-transgenic form and its frequency should decrease. If the converse is true, high benefits and low costs, the transgenic form is expected to increase in frequency. Moreover, the costs of transgene expression by commercial varieties are expected to be small as a result of strong artificial selection for production efficiency in commercial cultivars [8].

In ecological and evolutionary studies, traits are deemed beneficial when they promote the survival and reproduction of their bearers. For example, among the plant-incorporated proteins (PIPs), the family of *Bt* transgenes indirectly increases survival and reproduction by limiting damage by herbivores. Reduced herbivory often results in substantial increases in plant biomass and seed output

(see [10] for review). Changes in viability and fertility could have impacts on the population biology of natural populations; should resistant plants be more likely to survive, grow larger and produce more flowers. It follows that the frequency of transgenic resistance in the population would increase under selection [11]. Therefore, plants carrying a PIP for herbivore resistance, by their larger size and greater flower production, could sire more offspring than non-*Bt* forms.

We report herein our investigations of the effects of a genetically engineered herbivore resistance on crop and wild *Brassica* species and their hybrids under selection by herbivores and competitors. We grew transgenic, insect-resistant canola, non-transgenic canola, and *B. rapa* L. (Brassicaceae) with their hybrids in competition with congeners, or with common ruderal species in of the western U.S. In addition, we challenged these populations with diamondback moth (*Plutella xylostella*) (Lepidoptera: Plutellidae), a *Bt*-susceptible herbivore that is specialized on brassicaceous species. Our aims were to test the following predictions:

(1) the transgene will be beneficial in the presence of herbivores;

(2) the *Bt Cry1Ac* transgene is costly in the absence of herbivores, and

(3) selection by herbivores for insecticidal transgenes will increase the rate of transgene flow.

2. Results and Discussion

2.1. MANOVA Effects by Year and Univariate ANOVA Effects by Year and Genotype

Since the levels of herbivores applied were different in each year, it is necessary to analyze the data by Year. In the MANOVA by Year, Genotype had significant effects on aboveground biomass (ABM) and seed production (ESN) in both years of the study (Table 1). The main effects of Herbivory and Competition on ABM and ESN were significant only in the second year (Table 1). Interaction effects were significant for Genotype X Herbivory for ABM in 2007, ESN in 2006, and ESN in 2007. Finally, the interaction effects of Genotype X Competition was significant for ESN in 2006 and 2007 at the 0.10 level of significance (Table 1). Because genotype so clearly impacted the other effects of Herbivory and Competition, univariate ANOVA was performed by Year and by genotype (Table 2). In this analysis, Herbivory significantly impacted the different traits, though only in the year with higher herbivore density; 2007. Herbivory impacted the ABM measures of *B. rapa* and the non-GM F_1 in 2007, and the ESN measures of the GM F_1, *B. rapa*, non-GM F_1 and non-GM Westar genotypes. Competition effects were more variable by Year and significantly impacted genotypes in no discernable pattern (Table 2). The Herbivore X Competition interaction was not significant for any genotypes in either year. Details of the analyses and their impact on different measures of community performance are discussed below.

Table 1. MANOVA by Year for aboveground biomass (ABM) and estimated seed number (ESN) for experimental genotypes (G) growing under herbivory (H) and Competition (C) treatments. The effects of Herbivory, Competition and their interaction were analyzed at the level of the mesocosm as between-subject factors using the whole-plot error term for a split-plot design. The effects of Competition, Genotype and their interactions with Herbivory treatment were tested as within-subject factors at the tub level using the split-plot error term.

| 2006		ABM		ESN	
Between-subject factors	*df*	*F*	*p*	*F*	*p*
Herbivory (H)	1	0.94	0.370	2.76	0.148
Competition (C)	2	2.49	0.125	0.47	0.638
C X H	2	1.24	0.324	0.88	0.444
Within-subject factors					
Genotype (G)	4	475.19	<0.0001	130.40	<0.0001
G X H	4	0.68	0.625	3.62	0.067
G X C	8	0.72	0.673	2.76	0.047
G X C X H	8	0.35	0.931	1.37	0.291
2007		ABM		ESN	
Between-subject factors	*df*	*F*	*p*	*F*	*p*
Herbivory (H)	1	5.89	0.051	10.56	0.018
Competition (C)	2	3.57	0.061	2.65	0.115
C X H	2	0.10	0.908	0.65	0.540
Within-subject factors					
Genotype (G)	4	407.42	<0.0001	191.96	<0.0001
G X H	4	3.60	0.051	9.62	0.004
G X C	8	1.03	0.452	2.15	0.092
G X C X H	8	0.35	0.936	0.39	0.907

Table 2. Univariate ANOVA by Genotype, by Year, for ABM (g) and ESN (seeds) for experimental genotypes (G) growing under herbivory (H) and Competition (C) treatments with interactions (HxC). Significant effects shown in bold at a *p*-value of 0.1.

|		GM Westar		GM F1		*B. rapa*		Non-GM F1		Westar	
		2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
ABM	Herbivory (H)	0.537	0.720	0.391	0.863	0.727	**0.012**	0.927	**0.016**	0.613	0.587
	Competition (C)	0.718	0.867	0.278	**0.030**	0.711	0.235	**0.094**	0.194	0.532	0.397
	H X C	0.423	0.917	0.284	0.926	0.686	0.736	0.806	0.798	0.647	0.702
ESN	Herbivory (H)	0.191	0.248	0.108	**0.099**	0.307	**0.000**	0.970	**0.054**	0.813	**0.001**
	Competition (C)	0.861	0.850	0.344	**0.005**	0.229	0.615	**0.041**	0.228	0.835	0.143
	H X C	0.630	0.859	0.439	0.322	0.774	0.942	0.838	0.988	0.618	0.426

2.1.1. Heterosis

Genotype had a significant effect on ABM and ESN in both years (Table 1). F₁ hybrids were the largest plants in the study and *B. rapa* plants were the smallest (Figure 1). Both GM, and non-GM F₁ hybrid genotypes were significantly larger than the average *B. rapa* parent, and significantly larger

than their respective crop parent in 2007 (Figure 1). Contrast *p*-values are shown in Table 3. These results are largely consistent with previous studies reporting vegetative heterosis of F_1 hybrids between *B. rapa* and canola [8,12]. Regarding seed production, GM and non-GM F_1 hybrids produced significantly fewer seeds than the crop genotypes (GM Westar and Westar) demonstrating the negative fecundity effects of forming a triploid hybrid [13], but significantly more than *B. rapa* in both years ($p < 0.001$ for all contrasts) (Figure 2). This large reduction in seed production (relative to *B. napus*) has been observed in field examination of herbicide resistant hybrids produced from crop *B. napus* X wild *B. rapa* plants in Canada [13]. It is important to note that advanced backcrosses of these two species regain fecundity within a few generations.
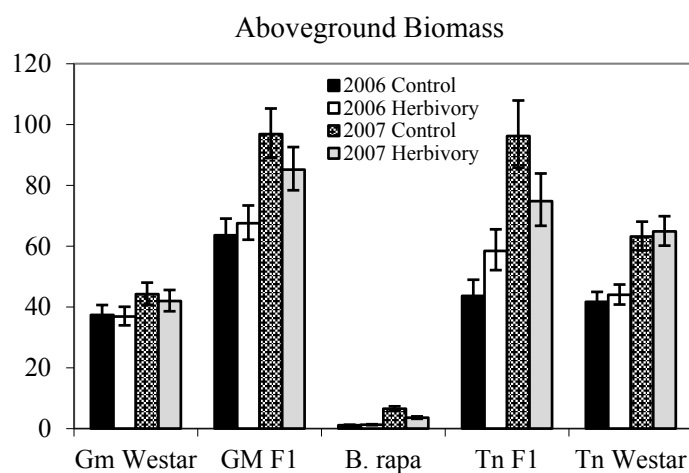
Figure 1. ABM of experimental genotypes growing under control and experimental herbivory treatments in 2006 (low herbivore densities) and 2007 (high herbivore densities). Bars represent mean (±1 SE). Closed bars = control; open bars = herbivory.
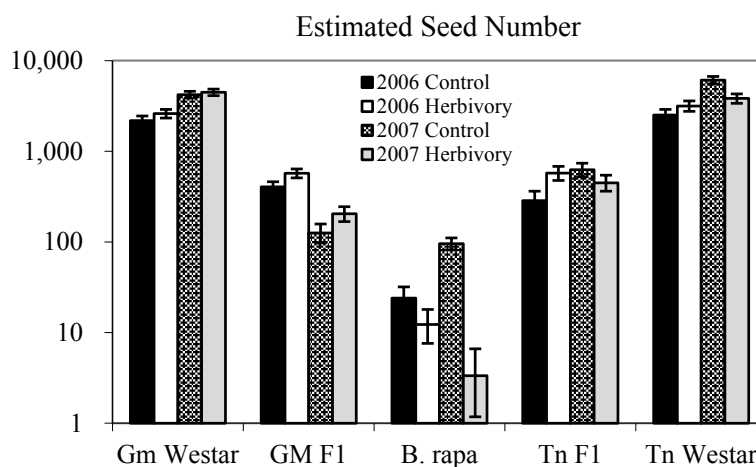
Figure 2. ESN of experimental genotypes under control and experimental herbivory treatments in 2006 (low herbivore densities) and 2007 (high herbivore densities). Bars represent mean (±1 SE). Closed bars = control; open bars = herbivory; *Y*-axis displayed in log scale.

Table 3. *Post hoc* contrasts between genotypes for ABM (g) and ESN (seeds) in each year for experimental genotypes growing under herbivory and control treatments. Values in bold indicate a significant Bonferroni corrected *p*-value of 0.01.

Contrast	ABM 2006		ABM 2007		ESN 2006		ESN 2007	
	Control	Herbivory	Control	Herbivory	Control	Herbivory	Control	Herbivory
GM Westar *vs.* GM F1	**0.0039**	**0.0049**	**0.0001**	**0.0005**	**0.0003**	**0.0001**	**0.0001**	**0.0001**
GM Westar *vs.* non-GM F1	0.4526	**0.0049**	**0.0007**	0.0136	**0.0011**	**0.0001**	**0.0001**	**0.0001**
GM Westar *vs.* Westar	0.372	0.1771	**0.0034**	**0.01**	0.7	0.1849	**0.0011**	0.3396
GM Westar *vs. B. rapa*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0002**	**0.0001**	**0.0001**	**0.0001**
GM F1 *vs.* non-GM F1	0.0755	0.4247	0.9272	0.1862	0.3987	0.9542	**0.0054**	0.0212
GM F1 *vs.* Westar	**0.0069**	0.0232	**0.0003**	**0.0004**	**0.0003**	**0.0001**	**0.0001**	**0.0001**
GM F1 *vs. B. rapa*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0012**	**0.0001**	0.0219	**0.0001**
Non-GM F1 *vs.* Westar	0.8273	0.1249	**0.0081**	0.0715	**0.0013**	**0.0003**	**0.0001**	**0.0001**
Non-GM F1 *vs. B. rapa*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0002**	**0.0001**	**0.0015**	**0.0001**
Westar *vs. B. rapa*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0002**	**0.0001**	**0.0001**	**0.0001**

2.1.2. Herbivory

The MANOVA by Year indicated that Herbivory significantly reduced ABM and ESN of the mesocosms in 2007, while Genotype X Herbivory terms had significant effects on ABM in 2007 and ESN in both years (Table 1). The significant main and interaction effects seen in 2007, relative to 2006 suggest that the difference in herbivore densities between 2006 (low densities) and 2007 (high densities) may have contributed to the changes observed. Given the dramatic visual observations of herbivory damage in the mesocosms in 2007, we believe herbivore density was largely responsible for the significant effects observed in that year although we are unable to rule out additional effects due to year.

The MANOVA analysis by Genotype shows how resistant and non-resistant genotypes responded to Herbivory in each year. Neither the GM Westar commercial line nor the GM F_1 hybrid line showed an effect of Herbivory on ABM measures in either year. Similarly, ESN measures were not significantly changed in 2006 for these genotypes, though a significant effect on ESN (increased seed number) in the GM F_1 was observed in 2007. These results demonstrate a general resistance of these genotypes to the applied stress and in the case of the GM F_1, increased fecundity. In contrast, non-GM lines (*B. rapa*, non-GM F_1, Westar) showed a significant effect of Herbivory for both response variables in 2007 with a no effect in the first year. These results mirror those observed for this transgene in field based experiments of Sunflower (*Helianthus annuus*) where transgenic plants were more resistant to lepidopteran herbivores [14].

Fertility and biomass of susceptible crop genotypes (*B. rapa*, non-GM F_1 hybrid, Westar) tolerated herbivory in 2006, but showed reduced growth and productivity in 2007 (Table 2). In contrast, the resistant, transgenic crop genotypes (GM Westar, GM F_1 hybrid) tolerated herbivory and showed no reduction of growth or fertility in the presence of diamondback moth in either year of the study.

Brassica rapa responded similarly to the herbivory treatment in both years of the study. Seed counts for *B. rapa* in herbivore-treated relative to control mesocosms were reduced by 29% in 2006, and 65% in 2007 (Figure 2). Furthermore, *B. rapa* biomass was significantly less in the herbivore treatment in 2007 ($p = 0.012$), but not in 2006. Overall, the effects of herbivory on *B. rapa* were more substantial than for any other genotype in the study, including the other vulnerable genotypes (non-GM F_1 and Westar).

2.1.3. Costs of *Bt Cry1Ac* Gene

In 2006, the genetically modified (GM) Westar canola growing in the herbivore-free mesocosms produced was smaller and produced fewer seeds than the non-GM Westar, though not significantly so ($p = 0.372$; $p = 0.700$). However, ABM and ESN measures in 2007 was significantly lower for the GM Westar in this contrast ($p = 0.003$; $p = 0.001$) *versus* the non-GM crop. As control mesocosms are not confounded by year or herbivory effects, we interpret this to indicate that constitutive expression of the inserted transgene, or the physical effects of transformation (e.g., site of insertion) of the Westar line may have a detrimental effect on the GM Westar in the environmental and competitive conditions of our mesocosm greenhouses. As a result, GM plants in this study might be expected to be smaller and produce fewer seeds than non-GM plants, all else equal. Costs of genetic engineering have not previously been reported for these lines [15] or others [8,13] in the *Brassica* system, nor in Sunflower [14]. They were observed here perhaps because of the stresses imposed by the mesocosm environments [16]. Similar observations of fitness costs are not uncommon and have been observed in other studies, for example, studies on herbicide resistance traits (reviewed by [17]). In these studies, fitness costs become measurable when variable stress is included as a factor in experimental designs. Detection of costs may have important implications for risk assessment and these results therefore merit further study.

The above results indicate that the costs and benefits of insecticidal transgenes vary in space and time. As demonstrated with the *B. napus* genotypes in the second year of the study, but not the first, the costs of genetic modification were overcome by benefits of reduced herbivory. The result was a net benefit of the transgene when herbivores were present. As a result, increases in biomass and fecundity associated with the transgenic phenotype may translate to fitness benefits of crop canola and its hybrids growing outside of cultivation. Therefore, environmental variation is expected to be an important factor determining whether a transgene or any other beneficial domesticated allele will pose a risk [8,18].

2.2. Competition

The main effect of Competition in the overall MANOVA was significant for ABM and ESN (Table 1) in 2007, but not in 2006. In general, all plants tended to be largest under control conditions and smallest in competition with ruderal weed species (data not shown). However, when analyzed independently, genotypes differed in response to competition: parental genotypes (*B. rapa*, GM Westar and Westar) showed no significant effect of Competition for either response variable. In contrast, the effects of competition treatments were significant for hybrids. ABM and ESN differed significantly

among competition treatments for the GM F_1 (ABM $p = 0.030$; ESN $p = 0.005$) in 2007 and non-GM F_1 genotypes in 2006 (ABM $p = 0.094$; ESN $p = 0.041$).

Further, *post hoc* analysis showed that both F_1 genotypes were smallest (ABM) when grown with ruderals relative to control competition, and competition with congeners (GM F_1 in 2007: $p = 0.034$, $p = 0.013$; non-GM F_1 in 2006: $p = 0.044$, $p = 0.086$, respectively). In addition, competition had an effect on ESN for the non-GM F_1 genotype in 2006 ($p = 0.041$). Non-GM F_1 lines produced significantly fewer seeds in ruderal relative to control treatments in 2006 ($p = 0.033$). The effects on the non-GM F_1 were not significant after Bonferonni adjustment nor were the effects on the GM F_1 ESN but the trend is worth noting given the restricted sample sizes used here. As there was no significant Herbivory X Competition interaction, effects are averaged over herbivore treatment.

Canola and F_1 hybrids are potentially aggressive competitors of *B. rapa* in the wild. Crops and crop-weed hybrids are physically large, with hybrids up to 50× the size of the *B. rapa* parent and larger even than the crop parent. However, we found little effect of the competition treatments on *B. rapa* or it was similarly affected by all treatments. Design limitations precluded additional competition treatments, but future studies should include a treatment of *B. rapa* in the absence of competition from crop and crop-weed hybrids.

2.3. Transgene Flow under Selection

All the *B. rapa* plants that survived to the end of the study and that produced seeds produced at least one transgenic F_1 hybrid seed. Gene flow rates, estimated as the proportion of *GFP+* progeny produced by each individual of *B. rapa*, ranged from 0%–100%. A significant increase in mean transgene flow rate was observed in the second year of the experiment (from 5.1% to 22.3%) ($\chi^2 = 3.87$, *df* = 1, $p = 0.049$). The cause of this increase is uncertain, but in neither year of the study did we detect a significant effect of herbivory on transgene flow rate.

High rates of outcrossing in *B. rapa* may be related to its breeding biology. *Brassica rapa* is self-sterile and obligately-outcrossing. Because *B. rapa* must outcross, the frequency of hybrids in a population will be a function of the availability of congeneric pollen in the pollen cloud [19]. The larger the canola population, the greater the potential hybridization rate and fewer the proportion of pure *B. rapa* seed in each successive generation [19]. The implications for this result are compelling, but additional studies of free-living populations are necessary to confirm these largely theoretical predictions.

3. Materials and Methods

3.1. Experimental Design

Fitness associated traits of transgenic, non-transgenic plants and their hybrids under selective pressure of an herbivore were evaluated in experiments carried out at the US EPA facilities in Corvallis, OR, USA. Experiments were performed during the springs of 2006 and 2007 in eight free-standing, outdoor mesocosms (detailed in [19]) using a split-plot design to test the effects of herbivory and intra-genotype competition on the vegetative and reproductive responses of five *Brassica* genotypes including *Brassica napus* L. (canola) and *Brassica rapa*. Each mesocosm was approximately

3.1 m diameter × 3.3 m height, with three large tubs each containing approximately 1 m³ of sandy loam soil. Four of the eight mesocosms were randomly assigned to the herbivory treatment (*i.e.*, presence of herbivore) and the other four mesocosms were assigned to the control (*i.e.*, absence of herbivore). *Plutella xylostella* L. (Diamondback moth) larvae were added to the treated mesocosms to simulate herbivory under field conditions. Measures of Diamondback moth (DBM) densities in the field range from 1–100 larvae per plant [20]. DBM densities in in 2006 were approximately 100 larvae per tub; approximately 6 per plant to simulate low selective pressure. In 2007, densities were approximately 2000 per tub; approximately 100 larvae per plant to simulate high selective pressure. To promote pollination and prevent pollen limitation, approximately 1000 *Musca domestica* L. (housefly) larvae were introduced to each mesocosm each year of the study as soon as the first flowers were evident (following [21]). Three individuals of each of five plant genotypes were planted in each mesocosm tub in a grid pattern to simulate a plant community. Within a mesocosm, each tub was randomly assigned to one of three competition treatments: no additional plant competitors ("control"), competition with additional *B. rapa* plants, or competition with ruderal species. To examine intra-generic competition effects, nine individuals of *B. rapa* were planted in and around the original planting grid. In the third treatment, three individuals of each of three ruderal species were planted within and around the experimental plants. Ruderal species were chosen to represent broadly distributed weeds of the western U.S. common on disturbed soils such as fallow fields and road verges: *Achillea millefolium* L. (common yarrow), *Panicum capillare* L. (witchgrass) and *Lapsana communis* L. (nipplewort). Each mesocosm was enclosed with 8 mil PVC film, exposed to ambient light, and regulated by evaporative coolers to approximate ambient temperature. Mesocosms were equipped with pollen filters and insect netting to prevent escape of *Brassica* pollen and seeds and to prevent movement of pollinators between units.

Seedlings were started in greenhouses, marked individually and transplanted at the four-leaf stage. Plants were grown under ambient light from mid-April to mid-July in 2006, and from mid-March to mid-July in 2007. When plants began to senesce, irrigation was stopped and plants were allowed to desiccate. Aboveground biomass was harvested two weeks later and dried in a forced air oven at 60 °C for 10 days. Seed numbers were estimated from the mass of 100 seed counts. All *B. rapa* seeds were screened for *GFP* expression. Transgenic hybrids were identified in qualitative assays for *GFP* fluorescence using 10× microscopy under long-wave ultraviolet light [22]. Gene flow rates (% transgenic seeds per *B. rapa* plant) and the total numbers of transgenic seeds produced per mesocosm were recorded.

3.2. Plant Materials

The five *Brassica* genotypes used in these experiments represent three parental lines: (1) *B. rapa*; (2) non-transgenic *B. napus* (canola) variety Westar (hereafter, "Westar"); and (3) transgenic Westar (hereafter, "GM Westar"); and two hybrids: (4) *B. rapa* X Westar (hereafter, "non-GM F_1"); and (5) an F1 hybrid-*B. rapa* X GM Westar (hereafter, "GM F_1"). *B. rapa* is a naturalized weed introduced from Asia, nearly cosmopolitan in the New World and frequently found in disturbed habitats (http://plants.usda.gov/java/). The species is considered a noxious weed in parts of the U.S. and an agronomic pest in Canada [23]. *Brassica rapa* L. is considered self-sterile, although evidence of

minimal selfing has been reported [18]. *B. rapa* seeds used in these experiments were collected from a persistent population on the campus of Oregon State University, Corvallis, OR, USA. *B. rapa* spontaneously hybridizes with *Brassica napus* L. (canola) in the field [22] at rates ranging from 0%–55% in field experiments and greenhouse studies [24–26]. F$_1$ generation hybrids with canola are triploid and vegetatively vigorous, but often express reduced fertility relative to the weedy or native parent species [13,15]. However, one study suggests that fertility is quickly reestablished in early backcross generations [8].

Brassica napus (canola) is an oilseed crop grown extensively in regions of the northern U.S. and southern Canada. Canola is an allotetraploid hybrid of *B. oleracea* L. and *B. rapa*, is predominantly self-fertile, but is sexually compatible with all cole crops and with a number of species listed by the USDA as noxious weeds (*B. rapa*, *Sinapis arvensis* L., *Erucastrum gallicum* (Willd.) O.E. Schulz) [24–26]. Canola is considered an annual, but secondary seed dormancy has now been confirmed [27]. Mature plants can overwinter as rosettes [17] and volunteers are routinely reported years after a canola harvest [21]. A substantial portion (1%–30%) of each crop is lost during harvest [27,28] and mature crops are frequently left unharvested because of unfavorable harvest conditions [29]. Overwintering and seed dormancy increase the phenological overlap with sexually compatible relatives thereby improving the likelihood of crop-weed hybridization [23].

The canola variety "Westar" was genetically modified with markers useful in the tracking of pollen movement and transgene flow [15,18]. Three transgenes were under the control of the CaMV 35S promoter in separate cassettes on a single T-DNA vector: *mgfp5-er* and a synthetic truncated *Bt cry1Ac* [12]. The pSAM12 plasmid (described in [30]) used to transform *B. napus* contained *GFP*, *Bt*, and kanamycin resistance cassettes in the T-DNA, enabling all three traits to be inserted in a single, genetically linked locus [12]. The *GFP* and *Bt* transgenes were shown to be genetically linked and functional through the BC4 generation [31]. This experimental transgenic Westar line was generated in the lab of Dr. Charles Neal Stewart, Jr., and is used with his permission. The GM Westar line selected for these experiments was shown through a series of selfed generations to be homozygous for all transgenes. Hybrid lines were developed by manual pollination under greenhouse conditions following Bautista *et al.* [32]. Eighteen *B. rapa* plants were hand-crossed on sequential days with pollen from GM Westar and Westar to generate F$_1$ hybrid generations. The hybrid status of a subset of individuals was verified with flow cytometry [33] (data not shown).

3.3. Statistical Analysis

Measures of vegetative biomass (ABM) and estimated seed number (ESN) were analyzed using multivariate analysis of variance (MANOVA) by Year with Genotype as the within-subject factor and Herbivory and Competition as the between-subject factors. It was not logistically possible to test different levels of herbivores in the same year due to limitations on the number of available mesocosm testing greenhouses. Because herbivore densities were different in 2006 and 2007, herbivory effects cannot be directly compared between years and were analyzed separately. While measurements were taken from individual plants, the main effect of Herbivory was analyzed at the mesocosm level using the whole-plot error term for a split-plot design. The main effect of Competition and its interaction with Herbivory were tested at the tub level using the split-plot error term. The MANOVA tests for

Genotype and its interactions with Herbivory and Competition were also performed at the tub level using the split-plot error term. When there were significant interactions, further analysis (ANOVA) was performed as on data subsets [34]. Since ABM and ESN measures differed by several orders of magnitude between genotypes, data were transformed prior to statistical analysis using the natural log transformation (ABM) and square root transformations (ESN). Values were back-transformed for reporting the results. Because the number of mesocosms limits the replication of the experiment, a *p*-value > 0.10 was deemed sufficient to detect significant effects. A one-sided *F*-test was used when testing for the effects of Herbivory. For *post hoc* contrasts between genotypes, a Bonferroni modified *p*-value was used ($p = 0.1/10$, contrasts = 0.01). The MANOVA and ANOVA analyses were generated using SAS/STAT software (PROC GLM) with planned contrast statements to examine differences between individual genotypes by Year. Wilcoxon rank sum test (exact) was used to test for differences between years and treatments in the frequency of transgene flow to *B. rapa*. We used mesocosm averages with SAS/STAT software (PROC NPAR1WAY), Version 9.13 of the SAS System for Windows (SAS 9.13, Cary, NC, USA).

4. Conclusions

This study examined the ecology of transgenic and non-transgenic *Brassica napus* crops and sexually compatible *Brassica rapa* weeds in an open pollination system competing with natives and congeners, and under selection by insect herbivores. The results suggest that canola, regardless of transgene phenotype, which has escaped from cultivation, has the potential to thrive in more naturalized landscapes. Moreover, crop-wild hybrids are able to compete effectively with both crop and wild congeners. These outcomes are more evident in the presence of a biotic stressor, the diamondback moth, differences in plant community composition and competition, and are impacted by the phenotype of herbivore resistance. Hybrid forms, whether they carried the transgene or not, had much greater vegetative and reproductive fitness traits compared with the wild/weedy *B. rapa*, demonstrating the potential for extirpation of *B. rapa* in populations where hybrid forms occur.

A recent review underscores the finding that few transgenes have found their way to native species or weeds [35]. Instead, transgene flow is reported more frequently among conspecific crops species where barriers to hybridization are limited. We suggest, however, that the long-term effects of agricultural species on native plant communities will require far more intense scrutiny as it is likely that rare events occur [36] and remain undetected for some time. The value of mesocosms studies such as these lays in the ability to safely simulate the conditions that could possibly promote the spread of a beneficial transgene and observing the initial shorter term responses at the population and community levels.

Author Contributions

Cynthia L. Sagers and Jason P. Londo conceived of the experiment. Cynthia L. Sagers drafted the manuscript. Jason P. Londo and Edward Henry Lee analyzed the data. Jason P. Londo designed the tables and figures. Nonnie Bautista developed the plant materials used in the experiment. Edward Henry Lee advised on the experimental design. George King and Lidia S. Watrud designed the planting arrays and managed the pre-submission review.

Conflict of Interest

The authors declare no conflict of interest.

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