

## Research Article

# Plant Fitness Assessment for Wild Relatives of Insect Resistant Bt-Crops

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When field tests of transgenic plants are precluded by practical containment concerns, manipulative experiments can detect potential consequences of crop-wild gene flow. Using topical sprays of bacterial *Bacillus thuringiensis* larvicide (Bt) and larval additions, we measured fitness effects of reduced herbivory on *Brassica rapa* (wild mustard) and *Raphanus sativus* (wild radish). These species represent different life histories among the potential recipients of Bt transgenes from Bt cole crops in the US and Asia, for which rare spontaneous crosses are expected under high exposure. Protected wild radish and wild mustard seedlings had approximately half the herbivore damage of exposed plants and 55% lower seedling mortality, resulting in 27% greater reproductive success, 14-day longer life-spans, and 118% more seeds, on average. Seed addition experiments in microcosms and *in situ* indicated that wild radish was more likely to spread than wild mustard in coastal grasslands.

## 1. Introduction

Commercialized transgenic, insect resistant (IR) crops currently grown in the United States have virtually no wild relatives near production sites, thus ensuring that novel crop traits are unlikely to move into local wild gene pools. However, an assessment of the consequences of gene flow will be necessary in future deregulation decisions because most of the major and minor crops in the world either exist in the wild themselves or hybridize with wild relatives somewhere in their range [1–5]. Wild relatives of transformed plants that obtain IR traits through gene flow and introgression may be released from the pressure exerted by susceptible herbivores [6–14]. However, scant knowledge about the ecological factors that regulate the abundance, competitive ability, or geographic range of weeds limits our ability to predict whether novel plant defenses are likely to increase the weediness of wild crop relatives [14] or even whether herbivory has a negative or positive effect on plant growth and fitness [15–20]. Surprisingly, few tests have been conducted on the effects of herbivory on the spread of invasive plants [21, 22] or to quantify the effects of herbivory on plant vital rates [23].

Identifying and quantifying environmental risks associated with gene flow from transgenic crops is subject to methodological tradeoffs because of containment restrictions, especially for plant fitness effects, which require pollen production. Field tests with pollen-producing transgenic plants must be contained physically in cages or greenhouses or established at sites where wild relatives do not occur. Conditions in regions that have no natural populations of wild relatives may differ from areas of concern for hybrid formation in ways that affect the results, and therefore the relevance, of such field tests. An alternative method, used in this study, is to conduct tests *in situ*, where hybrids would be expected to occur spontaneously, by using herbivore protection/addition techniques on nontransgenic wild-type plants. This method could be adapted to test for fitness effects of IR transgenes in crop-wild hybrids worldwide, for example, the Bt-Brassica IPM programs in Asia and Africa (Grzywacz et al. [24]), and Bt-maize adoption in Mexico.

We present herbivore protection experiments using Bt sprays on two very different species in the Brassicaceae, wild radish (*Raphanus sativus* L., which occurs in California as a hybrid complex with *R. raphanistrum* L. [25]) and wild

mustard (*Brassica rapa* L.) as a method to assess potential fitness differences should crop transgenes from Bt cole crops (such as Bt canola, Bt cabbage, or Bt broccoli) introgress into wild populations occurring in the local mosaic of agriculture and coastal wildlands. The flora surrounding cole crop fields includes naturalized cabbage plants (*B. oleracea* L.), exotic wild radish, and wild mustards (*B. rapa* L., *B. nigra* L., and others), as well as native relatives. Using the two local Brassicaceae species that differ the most in their habits and characteristics, we compared relative levels of plant mortality, longevity, reproductive success, and total seed production per seedling of Bt-protected versus Bt-exposed wild mustard (*B. rapa*) and wild radish (*R. sativus*) in the three main habitats where they occur naturally: cultivated (disked) agricultural fields, uncultivated agricultural field margins, and nearby coastal grasslands (Figure 1). These habitats differ in resources and vegetational quality and may differ in patterns of gene flow, so that they represent the range of conditions under which wild relatives of Bt cole crops occur locally. To explore the link between reproductive output and population size, one needs to quantify the relationship between seed production and recruitment [26, 27]. Therefore, we also compared plant recruitment with seed addition experiments in field plots and in microcosms of disked fields and grassland.

Our experiments on wild radish and wild mustard were designed to detect plant population responses in complex habitats and determine (1) if protection from Bt susceptible herbivore damage would result in increased survivorship, longevity, and/or fecundity compared to plants incurring damage within the natural range occurring on local plants, (2) if tolerance to herbivory varies between wild plant species or among habitats that differ categorically in terms of plant resources and vegetational background (disked agricultural soil versus agricultural field margins versus natural vegetation), and (3) if seed limitation is a likely regulatory mechanism for either species in different habitats. The advantage of simulating plant protection conferred by transgenic traits in wild plants is the ability to include multispecies interactions that can alter fitness effects in ways that differ fundamentally from outcomes predicted by experiments with isolated plants, caged or greenhouse trials, or with artificial herbivory (see [28]).

Drawbacks include the adequate matching of expression levels, persistence rates, and target insects with applications of insecticidal simulants, and any fundamental differences between actual transgenic hybrids and wild-type experimental plants. We compare the results of this simulation technique to our previous studies and to studies conducted with transgenic plants by other researchers to gauge the usefulness of a field simulation method in informing risk assessments and regulatory decision making more generally.

## 2. Materials and Methods

**2.1. Study System.** We compared individual fitness parameters of wild radish (Brassicaceae: *Raphanus sativus* L.) in 2003-2004 (year one) and wild radish and wild mustard

(Brassicaceae: *Brassica rapa* L.) in 2004-2005 (year two). In the California central coast region, both species are naturalized winter annuals, emerging as seedlings in October or November with the first rains. Wild mustard flowers earlier and produces seed in spring compared to radish, which flowers and produces seed through August or September. Occasionally, wild radish plants persist for two years. Both wild mustard and wild radish are self-incompatible, insect-pollinated, and belong to crop-weed-wild complexes [29, 30]. *R. sativus* and *B. rapa* have persistent seed banks and are common in agricultural fields, field margins, and coastal grasslands. These habitats differ in resources and vegetational quality. Agricultural habitats are commonly fertilized and irrigated, whereas adjacent grassland soils receive water only through seasonal rainfall. The highest disturbance levels are found in cultivated fields, which are disked to remove weeds and loosen soil before planting annual crops. Wild radish and wild mustard are treated with herbicides or controlled through machine or hand weeding by local growers and managers of nature reserves, railroad corridors, state and city parklands, and wetlands.

Wild radish and mustard host a variety of herbivores, including cabbage aphids (*Brevicoryne brassicae* L.), green peach aphids (*Myzus persicae* L.), flea beetles (*Phyllotreta cruciferae* L.), diamondback moths (*Plutella xylostella* L.), cabbage butterflies (*Pieris rapae* L.), cabbage loopers (*Trichoplusia ni* L.), slugs, and snails. We used weekly bacterial insecticide (Bt) spray to prevent tissue removal by susceptible lepidopterans such as *P. rapae* and thus create an herbivore exclusion treatment against some of the insect species that feed on these plants. All plants were sprayed weekly with either a suspension of 1 g Dipel Dry Flowable (DF) powder per 750 mL of deionized water (protected plants) or a suspension of the same concentration of deactivated Bt (exposed plants) as a control for added water and bacterial material. This type of Bt is an appropriate simulant for Cry1s, which are relevant for herbivores of *Brassica* spp. and their relatives. Frequent treatments are necessary to simulate persistent dosage levels of Bt transgene expression or to simply act as an herbivore exclusion treatment, because this microbial insecticide is broken down by UV radiation and washed off with rain; this method may underestimate the insecticidal effects of transgenes in geographic locations with high precipitation.

We added a single 1st or 2nd instar *Pieris rapae* L. (Family: Pieridae), reared from field-collected adults, at monthly intervals to each of the plants that were exposed to all herbivores. In coastal California, *P. rapae* adults are in flight year-round, ovipositing on host plants in the Brassicaceae (Letourneau pers. obs., [31]). Details of these treatments were based on data from three previous experiments. First, our laboratory trials showed that *P. rapae* larvae in Petri dishes on cabbage leaf disks sprayed with denatured Bt (control,  $n = 10$ ) fed and survived, whereas movement of larvae on disks with active Bt ( $n = 10$ ) rapidly ceased, followed by death. Product breakdown during drying and transport of sprayed foliage prevented us from determining and comparing Bt levels with the range of expression in transgenic plants. Second, cumulative samples in June–August, 1987,

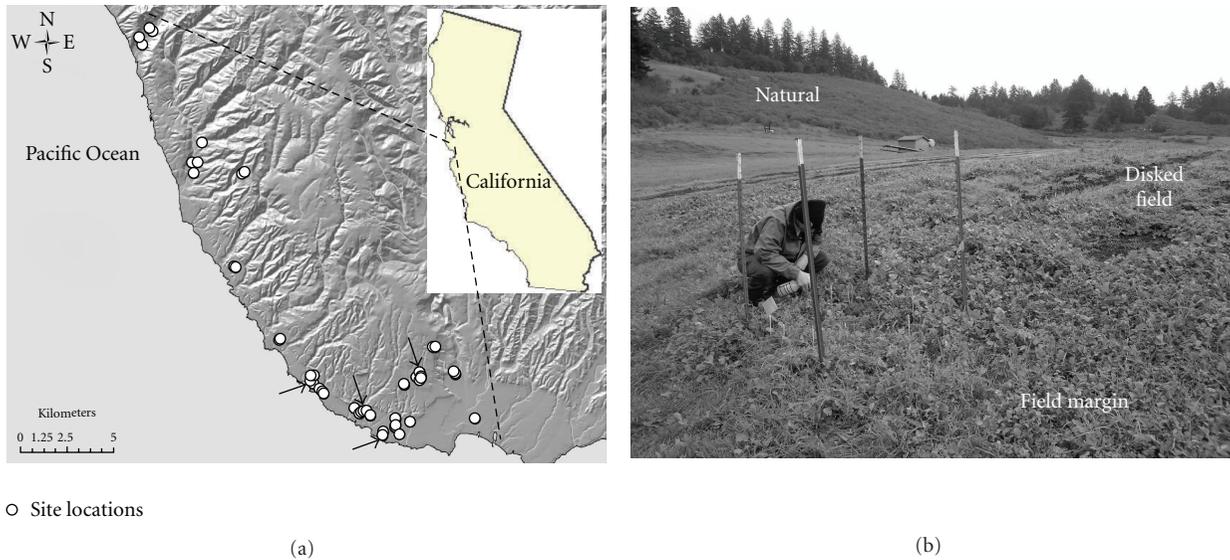


FIGURE 1: (a) Topographic map of California central coast, primarily Santa Cruz County, showing locations of four sites (Elkhorn Slough site was ~50 km south) used in year one (arrows) and of 90 1 m × 1 m plots established as 30 triplets of disked, field margin, and natural vegetation habitat plots in year two (GPS locations shown as white circles with black borders). (b) Example of disked field plot in year two, with T.R.F. Roubison spraying individual wild radish and wild mustard plants with Bt and denatured Bt amongst other forbs and grasses that emerged from soil seedbanks with the winter rains. Not shown are the associated field margin plot, which was within 5 m of the field, and the natural vegetation plot in uncultivated lands as shown in the distance.

a nonoutbreak year for lepidopterans, showed an average density of three lepidopteran eggs per *B. rapa* plant in unfertilized control plots within the study area for the current experiments [32, 33]. Finally, there were high levels of variability in ambient herbivory on *R. sativus* plants among sites and sample dates in preliminary field trials with 80–100 plants per habitat type at two sites in 2002 (0 to 100% per plant). The average number of damaged leaves in Bt-sprayed plots ranged from 8% to 55% of those damaged in ambient herbivory control plots (no Bt spray, no added larvae).

**2.2. Study Sites.** Experiments were conducted in the central coast region of California (36.974°N, –122.029°W), where 780 mm annual precipitation in this Mediterranean climate falls between October and May. In 2003–2004 (year one), sixty 1 m × 1 m field plots were established at each of five sites (Figure 1(a), arrows): the research farm at the University of California Santa Cruz Center for Agroecology and Sustainable Food Systems (UCSC CASFS), the UC Younger Lagoon Natural Reserve, east and west sites at Wilder Ranch State Park, and the Elkhorn Slough National Estuarine Research Reserve (not shown, 40 km south). These sites differ in historical land use, dominant vegetation, and local climate. In year two (2004–2005), three 1 m × 1 m experimental plots were established at each of 30 field sites along ~35 km of coastal Santa Cruz County, CA. In both years, one-third of the plots were designated for each of three habitat types (Figure 1(b)). Disked field habitat plots, located on the edge of an agricultural field (≤5 m from row crops), were dug with a shovel to turn over 10–20 cm of soil to simulate disking and disrupt any vegetation formerly

occurring on that plot. Field margin habitat plots were not disked or shoveled and had weedy vegetation, often on soil compacted by farm machinery. Both disked field and field margin plots were exposed to rain and irrigation run-off from fertilized fields (downhill placement from adjacent farm fields). Natural vegetation plots were established away from agriculture, had a mixture of naturally occurring native and exotic plants, and received no run-off or irrigation. All plots were established where dried plants of wild radish or wild mustard (or both) were already present within the plot or nearby (within seed-rain distance from plot).

**2.3. Experimental Design.** Treatment design was hierarchical in year one, with herbivory treatments (two levels) nested in habitat type (three levels—except at two locations where seedlings were scarce in disked plots), nested in location (five levels). Ten naturally occurring wild radish seedlings were selected in each plot (2,600 seedlings in total) and marked initially with numbered stakes and then with a numbered spiral binding ring around the plant stem. Paired plots were randomly designated to receive either weekly Bt sprays (protected plants) or denatured Bt sprays and larval additions (exposed plants). A single early instar *P. rapae* was placed on each experimental wild radish plant at three evenly spaced intervals between December and February, corresponding to plant stages from seedling to reproducing. Percent herbivore damage per plot was estimated once, 12 weeks after the first rains, by assigning a damage category to each leaf of each plant (0%, 1–10%, 11–25%, 26–50%, >50%) and converting the average category level per plant to the mid-point percentage of that category (e.g., 18% for

11–25%). The total number of viable radish seeds per small plant was counted after lightly crushing pods. For large plants, counts of viable radish seed for  $\approx 15$  pods were used to estimate total plant number of seeds by weight.

In year two, experimental plots (one set of three habitat types at each of 30 sites, Figures 1(a) and 1(b)) were divided into four 50 cm  $\times$  50 cm subplots to accommodate herbivore protection and herbivore exposure treatments for both wild radish and wild mustard. Before the first rain, to supplement any seedlings emerging naturally from the seed bank, eight radish seed pods collected from 25 mother plants in year one were added to each radish subplot and approximately 20 mustard seeds from 10 mother plants were added to mustard subplots. We reseeded subplots after 10 days if fewer than six seedlings of either plant species emerged. Three weeks after the first rains, four wild radish and four wild mustard seedlings per subplot (total of 1,260 plants) were numbered individually, assigned to receive either Bt sprays or inactivated Bt spray and larval additions, and followed weekly thereafter. *P. rapae* larvae were added at 3–4 week intervals, for a total of two to four larvae per plant over the season (up to four on long-lived plants). Percent herbivory per seedling was estimated (using categorical estimates of 0%, 1–5%, 6–60%, 61–90% and >90%, and their mid-points for calculating average estimates), and seedling mortality rates were determined eight weeks after the first rain. Percent herbivory was estimated again 21 weeks after the first rains. Although seed in disked plots were sown into bare soil, field margin plots usually had some standing vegetation and natural vegetation plots had very high cover rates of dried grasses and other annuals as well as some green perennial plants. At approximately 12 weeks after the first rain, as experimental plants and other species began to bolt and flower, bare soil cover and species richness of plants within each subplot was estimated. Wild mustard and radish plant status (rosette, bolting, budding, flowering, producing siliques or pods, or dead) was recorded on two of the four plants at week 15 after the rains. We continued to record the status of those plants every two weeks through week 39 when all plants had either died or finished producing seeds. Seed counts and estimates were done as in year one.

**2.4. Seed Addition Experiments.** For an increase in seed output to result in increased spread of the population, any production of additional seed has to result in additional plants surviving to reproduce in the habitat. To assess the potential fitness advantage of an increase in seed output by wild radish and wild mustard, we tested for seed limitation in a microcosm experiment with two of the habitat types (disked field and natural vegetation). Before the first rains (August/September 2006), large soil cores (25.4 cm diameter), with the existing dry vegetation and seed bank completely intact, were transferred to pots with care taken to minimize disturbance to the plant cover or soil profile. Two pairs of these large soil cores were taken from each of the 10 sites along the central coast with recently tilled soil, with one of each pair of the soil cores taken from a disked farm field before crops were sown and one taken from nearby areas

(same soil type) with natural vegetation intact. To test for any impact of increased seed yield, eight wild radish pods (lightly crushed) yielding 4–10 seeds/pod and 15 seeds of wild mustard were added to one of each pair of soil cores in three-gallon pots representing either disked agricultural fields or grassland. The number of seeds added approximated one-half the increase in number of seeds produced by a single wild radish plant (36.5 seeds) or wild mustard plant (26.5 seeds) when that mother plant has been protected from herbivory (average of years one and two from our field results). The resulting potted microcosms were used to ensure that runoff from heavy rains would not cause unaccountable seed losses. Pots were transported to the rooftop greenhouse at UCSC where they could be watered biweekly as needed and exposed to coastal weather for 10 weeks until wild mustard in the pots produced siliques and wild radish plants produced pods. A small scale field experiment at two of our sites (UCSC CASFS and the Homeless Garden Project) was used to further test these seed additions *in situ* in disked field and natural vegetation plots over six months in 2008–2009. Rings from pots were used to maintain added seeds in five replicate plots per habitat at each of the two sites.

**2.5. Data Analyses.** All analyses used PC-SAS v. 9.1 (SAS Institute 1990). In year one, both the average herbivore damage at 12 weeks after the first rain and the lifetime seed output for wild radish seedlings (rank transformed) were compared with respect to (1) herbivory treatment (protected versus exposed), (2) habitat type (disked field, field margin, and natural vegetation), and (3) interactions between herbivory treatment and habitat type. We used a General Linear Models (GLM) nested ANOVA to test for any significant effects, designating the error term as the type III mean square value for plot nested in the interaction term for site by treatment by habitat type. In year two, we used a logistic regression to compare categorical estimates of herbivory after eight weeks (0%, 2.5%, 25%, 75%, 95%), plant mortality (dead or alive), and lifetime production of siliques/pods (reproductive or not). Independent variables were plant species (wild radish versus wild mustard), herbivory treatment (protected versus exposed), and habitat type (disked field, field margin, and natural vegetation). Additionally, we used a GLM repeated measures ANOVA to test for changes in herbivory with time and to test for seasonal effects of habitat type, plant species, and interactions among these factors. Mean seed output per plant per plot, which could not be transformed to meet the assumptions of normality, was compared using a GLM ANOVA on ranks. Similarly, percent cover of bare soil after community development for 12 weeks was compared among subplots with ANOVA on ranks. All subplots for wild mustard and wild radish, and both herbivory treatments, were pooled to test for differences among habitat types because there were no significant differences in bare soil cover due to plant species or herbivore treatment.

The number of reproductive wild radish and wild mustard plants in the potted seed addition experiment was analyzed separately by plant species, with  $n = 10$  microcosms for the factorial experiment with two levels of seed addition

(seed added versus no seed added) and two levels of habitat type (disked field versus natural vegetation), using GLM ANOVA on ranks because the data could not be transformed to approximate a normal distribution. For seed addition experiments in the field, the total number of wild radish plants and wild mustard plants in each plot ( $n = 5$  plots per plant per treatment per habitat type) were compared separately, six months after the first rain, using a nested GLM ANOVA with the error term for nesting within sites as a replicate (seed treatment by habitat by site) on ranks.

### 3. Results

**3.1. Herbivory.** In year one, 12 weeks after the first rains that caused seeds to germinate in experimental plots, juvenile wild radish plants protected with activated Bt sprays had an average of  $5.8\% \pm 0.4\%$  herbivory caused by slugs, snails, beetles, and possibly lepidopterans that are not susceptible to Bt toxins [32]. This level of herbivore damage was significantly lower than the average  $11.1\% \pm 0.8\%$  for unprotected wild radish plants exposed to all herbivores plus added *P. rapae* (ANOVA for treatment nested in site,  $F_{5,218} = 8.8$ ,  $P < 0.0001$ ). Herbivore damage was significantly greater in disked field plots ( $9.7\% \pm 0.6\%$ ) than in field margin and natural vegetation plots ( $7.8\% \pm 0.4\%$  and  $8.0\% \pm 0.4\%$ , resp.) (ANOVA for habitat type nested in site  $F_{8,218} = 2.4$ ,  $P = 0.0182$ ). Wild radish seedlings may have been more apparent to naturally occurring herbivores in disked plots free of other vegetation. Also, in the process of inoculating the plants in all habitats, we commonly observed spider predation on larvae; it is possible that such predation was more common when inoculated plants occurred in established vegetation. In all habitat types, however, protected plants were damaged significantly less than those exposed to all herbivores (no significant interaction between treatment and habitat type,  $P = 0.1872$ ).

In year two, eight weeks after the first rains, wild radish and wild mustard seedlings protected with Bt sprays again received approximately one-half ( $12.2\% \pm 1.7\%$  versus  $27.4\% \pm 2.6\%$ ) the amount of herbivory received by unprotected plants exposed to all herbivores plus *P. rapae* larvae (Figure 2). Seedling damage was significantly lower in natural vegetation plots than in field margin habitat plots, with neither natural nor field margin habitats differing from disked plots ( $23.1\% \pm 2.2\%$  in field margin versus  $17.1\% \pm 1.8\%$  in natural vegetation and  $20.8\% \pm 1.9\%$  in disked field plots), but this pattern was strongly expressed only in wild mustard and not in wild radish (Figure 2). The lack of a treatment by habitat type interaction indicates that protected plants suffered lower levels of herbivory than exposed plants in all habitat types. When most mustard and some radish plants were starting to flower, at 21 weeks after the first winter rain, mean damage levels per plant were  $5.0\% \pm 1.2\%$  versus  $9.9\% \pm 1.8\%$  for protected and exposed plants, respectively. Damage levels dropped significantly from eight to 21 weeks after the first rain, but only the herbivory treatment remained significant (no between subjects effect of species or habitat type, and

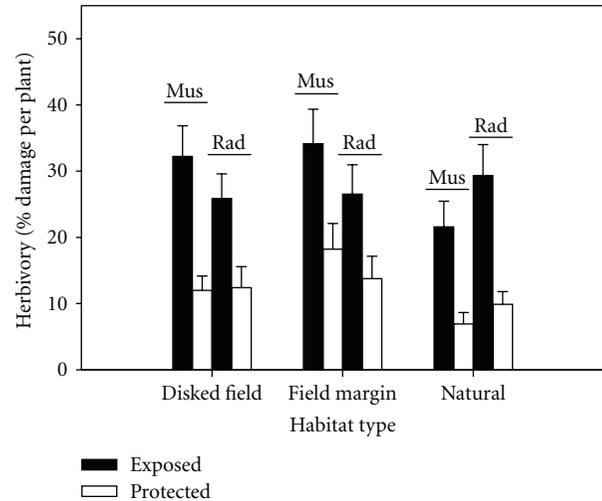


FIGURE 2: Year 2: mean ( $\pm 1$  SE) percent damage per plant per plot 8 weeks after rains on exposed versus protected [tmt] wild mustard and wild radish [species] in disked field, field margin, and natural vegetation plots [hab]. Wild mustard bars, labeled “Mus,” precede wild radish bars, labeled “Rad.” Logistic regression (using  $n = 30$  plot averages of 4 plants) showed significant effects for tmt ( $df = 1$ ,  $X^2 = 52.3$ ,  $P = 0.0001$ ), hab ( $df = 2$ ,  $X^2 = 6.6$ ,  $P = 0.0365$ ), and a nearly significant species\*hab interaction ( $df = 2$ ,  $X^2 = 5.6$ ,  $P = 0.0594$ ), but no significant effects for species, or species\*tmt, tmt\*hab, or species\*tmt\*hab interactions.

no within subject interactions among time, treatment, species, or habitat type) (repeated measures GLM: herbivory treatment  $F_{1,178} = 4.47$ ,  $P = 0.0359$ ). Lower herbivory levels later in the season indicated constant or lower herbivore pressure per unit tissue as plants grew larger or, especially for wild radish, an outcome of plants dropping damaged leaves.

**3.2. Plant Mortality, Vegetation Cover, and Reproductive Success Rates.** In year two, seedling mortality of wild mustard and wild radish was significantly lower for protected compared to exposed plants (Figure 3(a)). Exposed seedlings suffered 55% greater mortality than protected ones ( $17.8\% \pm 1.8\%$  SE versus  $11.9\% \pm 1.5\%$  SE). Seedling mortality was not significantly different among habitat types, though disked field plots tended to have lower seedling mortality rates overall (Figure 3(a)). There were no significant interactions involving habitat, herbivory, or species. The mortality rate of juvenile plants was 24% overall, with mortality rates not significantly different among plant species, treatments, or habitat types (Logistic regression, model value for Wald  $X^2 = 13.7$ ,  $P = 0.2528$ ), though mustard suffered higher juvenile mortality due to herbivory treatment than did wild radish (logistic regression for species by treatment interaction, Wald  $X^2 = 4.2$ ,  $P = 0.0413$ ).

Background vegetation had established fully by the time wild mustard and wild radish were reproductive, owing to the germination and growth of winter annuals after the fall rains began. By 12–13 weeks after the first rain, the average plant cover in all subplots was over 90%, including the disked field subplots, which initially had zero plant cover. The

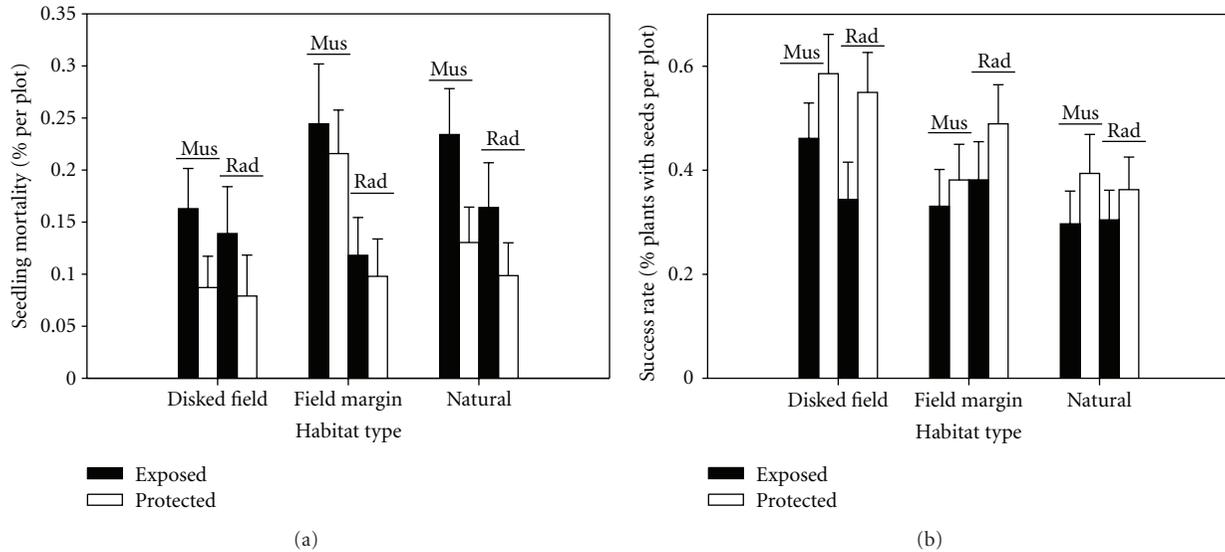


FIGURE 3: (a) Year 2: mean ( $\pm 1$  SE) percent seedling mortality per plot ( $n = 30$  plots with 4 plants each) in year two on exposed versus protected [tmt] wild mustard and radish [species] in disked field, field margin, and natural vegetation plots [hab]. Wild mustard bars, labeled “Mus,” precede wild radish bars, labeled “Rad.” Logistic regression showed significant effects for species ( $df = 1$ ,  $X^2 = 9.7$ ,  $P = 0.0019$ ) and tmt ( $df = 1$ ,  $X^2 = 6.2$ ,  $P = 0.0127$ ), but not strongly for hab ( $df = 2$ ,  $X^2 = 5.0$ ,  $P = 0.0826$ ), and none for any of the interactions: species\*hab, species\*tmt, tmt\*hab, or species\*tmt\*hab. (b) Year 2: mean ( $\pm 1$  SE) percent of plants per plot ( $n = 28$ – $30$  plots with 2 plants/plot) producing pods/siliques on exposed versus protected [tmt] wild mustard and radish [species] in disked field, field margin, and natural vegetation plots [hab]. Wild mustard bars, labeled “Mus,” precede wild radish bars, labeled “Rad.” Logistic regression shows significant effects for tmt ( $df = 1$ ,  $X^2 = 7.0$ ,  $P = 0.0082$ ) and hab ( $df = 2$ ,  $X^2 = 7.5$ ,  $P = 0.0237$ ), but not for species, species\*hab, species\*tmt, tmt\*hab, or species\*tmt\*hab interactions.

average plant species richness overall was  $6.8 \pm 0.1$  SE in all subplots, with no significant differences among habitat types. The average percent cover of bare soil in disked plots was not significantly different from that of field margin habitats but remained significantly greater than in natural vegetation habitats ( $10.5\% \pm 2.2\%$  SE in natural vegetation versus  $9.4\% \pm 1.8\%$  SE in field margins versus  $6.6\% \pm 1.7\%$  SE in disked field plots) (GLM ANOVA on rank percent bare soil cover  $F = 3.15$ ,  $P = 0.0450$ ).

A significantly greater percentage of protected wild radish and mustard plants produced siliques or pods ( $46.3\% \pm 3.0\%$  SE) than did exposed plants ( $36.5\% \pm 2.8\%$  SE), a 27% increase in reproductive success rate (Figure 3(b)). Plants in disked plots had significantly higher reproductive success rates than did plants in either field margin or natural vegetation plots, suggesting that greater plant competition or poorer resource conditions in the latter habitats made those habitats less favorable for wild radish or wild mustard reproductive success in general, without regard to herbivore pressure (Figure 3(b)). Wild radish is a longer-lived species than wild mustard (living for  $26.2$  weeks  $\pm 0.7$  SE versus  $18.3$  weeks  $\pm 0.4$  SE, ANOVA on ranked number of weeks,  $F = 96.3$ ,  $P < 0.001$ ), and plant longevity was increased overall by reducing herbivory (both species combined, no significant interaction between species and herbivory treatment). The average longevity for protected plants was  $23.1 \pm 0.7$  SE weeks versus  $21.4 \pm 0.6$  SE weeks for exposed plants (ANOVA on ranked number of weeks,  $F = 4.9$ ,  $P = 0.0276$ ). Neither habitat type nor any of the interactions among factors were

significant factors affecting plant longevity, and none of the interactions among herbivore pressure and species or habitat affected reproductive success (Figure 3(b)).

**3.3. Seed Production.** In year one, lifetime seed output of an average wild radish seedling, taking into account that early mortality results in zero seed production, was relatively low overall (Figure 4(a)) compared to year two in Figure 4(b)). Nevertheless, protected wild radish plants in the reduced herbivory treatment produced significantly more seeds, showing an almost twofold increase in fitness in terms of average seeds per seedling ( $14.8 \pm 2.3$  SE seeds) compared to exposed plants ( $8.4 \pm 1.4$  SE seeds). Average seed output per wild radish seedling was also significantly greater in disked and field margin plots ( $13.1 \pm 3.0$  SE and  $12.1 \pm 2.2$  SE, resp.) than in natural vegetation plots ( $10.5 \pm 1.5$  SE) (Figure 4(a)).

Similarly, in year two, protected wild radish and wild mustard seedlings produced significantly more seeds ( $100.6$  seeds/seedling) on average than exposed plants with higher levels of herbivory ( $46.1$  seeds/seedling) (Figure 4(b)), with no treatment by habitat or treatment by species interactions. Both wild mustard and wild radish responded similarly to the herbivory treatment, but wild mustard plants were more strongly responsible for a significant effect of habitat type (marginally significant interaction between species and habitat,  $P = 0.0627$ ). Mustard plants produced fewer seeds per seedling in natural vegetation plots than in disked or field margin plots, even when protected from lepidopteran

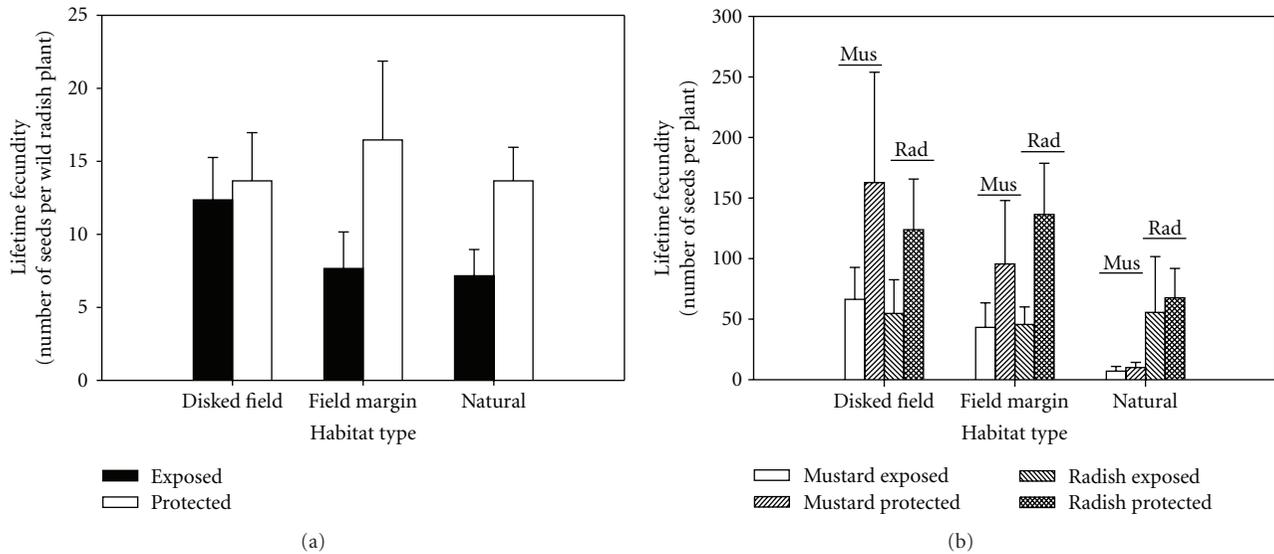


FIGURE 4: (a) Year 1: mean ( $\pm 1$  SE) number of seeds per plant per plot ( $n = 10$  plots with 10 plants each) produced by wild radish with two herbivory treatments (tmt: exposed to herbivores or protected from herbivores) in three habitat types (hab: disked field, field margin, or natural vegetation habitats); ANOVA on rank transformed levels of seed production per plant per plot showed significant effects for tmt (ANOVA for herbivory treatment nested in site,  $F_{5,232} = 4.9$ ,  $P = 0.0003$ ) and hab (ANOVA for habitat type nested in site,  $F_{8,232} = 6.7$ ,  $P < 0.0001$ ), with seed production greater on protected than exposed plants and in agricultural habitats than in natural vegetation, but no interaction between herbivory treatment and habitat type. Note that averages include plants that produced no seeds due to early mortality. (b) Year 2: mean ( $\pm 1$  SE) number of seeds per plant per plot ( $n = 28$ – $30$  plots with 2 plants per plot) on exposed versus protected [tmt] wild mustard and radish [species] in disked field, field margin, and natural vegetation habitats [hab]. Wild mustard bars, labeled “Mus,” precede wild radish bars, labeled “Rad.” ANOVA on rank transformed levels of seed production per plant per plot showed significant effects for tmt ( $F_{1,333} = 6.3$ ,  $P = 0.0127$ ) and hab ( $F_{2,333} = 3.7$ ,  $P = 0.0246$ ), where seed set was significantly lower in natural habitats than in agricultural habitats, but the vulnerability of wild mustard in this regard was shown by a marginally significant effect of species\*hab ( $F_{2,333} = 2.8$ ,  $P = 0.0627$ ), and no significant effects for species, species\*tmt, tmt\*hab, or species\*tmt\*hab interactions. Note that averages include plants that produced no seeds due to early mortality.

herbivory (Figure 4(b)). In a separate analysis of seed output *only* by those plants that lived to become reproductive and produce seed, radish produced significantly more seed than mustard (means of  $215.2 \pm 37.4$  SE and  $148.5 \pm 28.7$  SE, resp., ANOVA on ranks,  $F_{1,392} = 14.0$ ,  $P = 0.0002$ ), plants in disked and field margin habitats produced more than plants in natural vegetation habitats (means of  $214.6 \pm 40.5$  SE,  $210.7 \pm 43.2$  SE and  $106.3 \pm 36.0$  SE, resp., ANOVA on ranks,  $F_{1,392} = 8.0$ ,  $P = 0.0004$ ) but only marginally significantly more seeds when protected from lepidopteran herbivory ( $202.8 \pm 31.8$  SE and  $153.1 \pm 34.5$  SE per plant, resp., ANOVA on ranks,  $F_{1,392} = 3.1$ ,  $P = 0.0779$ ), with no treatment by habitat or treatment by species interactions.

**3.4. Seed Limitation.** Experimental addition of wild radish and wild mustard seeds resulted in higher recruitment, even when the number of seeds per microcosm or field plot was relatively low (estimated as 1/2 the additional seed produced by an average plant in the low herbivory treatment). Mean wild radish density was significantly greater when seeds were added to the soil compared to controls with no added seeds, whether in the disked soil microcosms (initially bare) or in the natural vegetation microcosms (with plant cover intact) (Table 1). Species richness of other plants emerging from the seed bank in disked soil microcosms averaged  $4.5 (\pm 0.3)$  SE

forbs and grasses, and natural vegetation microcosms had a mean of  $5.2 (\pm 0.4)$  SE other plant species, indicating that seed additions make a difference in recruitment of wild radish in the context of developing plant communities in these different habitat types. Seed addition also significantly increased mean recruitment of reproductive wild mustard plants (Table 1), but only in disked soil habitats. In contrast to wild radish, wild mustard density was significantly affected by habitat type (disked versus natural), such that few plants survived to flowering in natural vegetation microcosms. Species richness of other plants in these microcosms averaged  $4.6 (\pm 0.3)$  SE and  $5.3 (\pm 0.3)$  SE other forbs and grasses in disked and natural vegetation microcosms, respectively.

Seed additions carried out at two field sites showed that six months after the first rains, the number of wild radish plants established in field plots was (1) significantly greater when seeds were added than in control plots with no seeds added and (2) significantly greater in disked fields than in grasslands (Table 1). Seed addition produced the same result in disked fields as grasslands, as indicated by the lack of a treatment by habitat interaction. Similarly, the number of wild mustard plants was significantly increased by seed addition in disked field plots compared to controls, and disked plots had significantly more mustard plants than natural vegetation plots (grasslands). However, a significant

TABLE 1: Mean number of radish or mustard plants surviving to reproduce when the approximate half the mean seed output advantage per plant due to herbivore protection was added to microcosms ( $n = 10$  per habitat type) and field plots ( $n = 5$  per habitat per field site) compared to microcosms and field plots to which no seeds were added (control). Note that some seeds (whether added or present in the seed bank) exhibit dormancy.

Experiment	Disked soil habitat		Grassland habitat	
	Seeds added	Control	Seeds added	Control
Radish microcosms <sup>1</sup>	7.4 + 0.1	0.3 + 0.2	7.9 + 2.6	3.9 + 1.1
Mustard microcosms <sup>2</sup>	3.2 + 0.7	0.0 + 0.0	0.7 + 0.3	0.0 + 0.0
Radish field plots <sup>3</sup>	2.0 + 0.8	0.1 + 0.07	4.7 + 1.95	3.6 + 1.03
Mustard field plots <sup>4</sup>	7.0 + 1.96	1.1 + 0.68	0.0 + 0.0	0.0 + 0.0

<sup>1</sup>ANOVA on rank number of flowering radish plants per microcosm, with  $F = 30.5$ ,  $P = 0.0001$  for seed addition effects,  $F = 1.4$ ,  $P = 0.2452$  for disked versus natural soil microcosms, and  $F = 6.7$ ,  $P = 0.0136$  for seed addition by habitat interaction.

<sup>2</sup>ANOVA on rank number of flowering wild mustard plants per microcosm, with  $F = 26.6$ ,  $P < 0.0001$  for seed addition effects,  $F = 7.4$ ,  $P = 0.0101$  for disked versus natural soil, and  $F = 4.2$ ,  $P = 0.0485$  for seed addition by community interaction.

<sup>3</sup>Nested ANOVA on ranks for the number of wild radish plants after six months, seed addition treatment (site),  $F_{2,56} = 4.72$ ,  $P = 0.0165$ ; habitat (site)  $F_{2,56} = 23.13$ ,  $P \leq 0.0001$ ; treatment\* habitat (site)  $F_{2,56} = 1.63$ ,  $P = 0.2125$ .

<sup>4</sup>Nested ANOVA on ranks for the number of wild mustard plants after six months, seed addition treatment (site),  $F_{2,56} = 8.0$ ,  $P = 0.0123$ ; habitat (site)  $F_{2,56} = 8.24$ ,  $P = 0.0114$ ; treatment\* habitat (site)  $F_{2,56} = 8.0$ ,  $P = 0.0123$ .

interaction between seed addition treatment and habitat for wild mustard densities showed that seed addition effects were limited to disked plots, consistent with results of the microcosm experiment (Table 1). The impacts of seed addition on density of wild radish and wild mustard measured in these experiments are conservative, as a high proportion of seed for both species maintain dormancy so that seed addition can have a larger impact over time than that measured within a single season, such as in our experiments.

## 4. Discussion

**4.1. Herbivory and Plant Fitness.** Although there is general agreement that herbivores can have pronounced negative effects on plant fitness, plants also exhibit resistance to herbivores and tolerance to herbivore damage [34]. Thus, simple field tests have a place in directing further study and consideration of mitigation techniques toward the cases of expected transgene introgression which are most likely to increase the fitness and spread of weedy wild relatives in natural plant communities and agroecosystems (see Kwit et al. [35]). In our study, slight-to-moderate protection from herbivory, measured as the average difference between treatments on juvenile plants (5% on wild radish in year one, 17% on wild mustard in year two, and 16% on wild radish in year two), significantly increased lifetime seed output of an average plant to about double that measured for plants with greater mean herbivory. Typical fluctuations in herbivory,

then, that routinely reduce plant fitness in the field under present conditions, may be nullified to a significant degree with the introgression of a Bt trait for antiherbivore defense. The result could cause average seed output to shift to a higher level, depending on population levels of susceptible herbivores. A substantial proportion of plants protected in our treatments suffered some amount of foliar tissue removal (e.g., 45% of Bt sprayed wild mustard in 2003–2004) suggesting that both Bt susceptible and unsusceptible invertebrate species consumed plant tissue.

Lepidopteran resistant Bt-canola (*B. napus* L.), and possibly Bt broccoli or cabbage, would likely hybridize with *B. rapa* and other wild relatives if grown commercially in coastal California [36–40]. To test for potential fitness effects in two contrasting wild relatives in this study, we selected the two most abundant weedy relatives near cole crop fields, *B. rapa* and *R. sativus*, which differ in most life history traits. Although little is known about *R. sativus*, tests with *R. raphanistrum* show that it is unlikely to hybridize with *Brassica* crops (but see Gueritain et al. [41]). Because crop-to-weed gene flow is highly idiosyncratic and difficult to preclude totally in many regions of the world and for many crop plants, research on the effects of transgenes on natural communities ([42] and references therein) and field tests where wild relatives of pest-resistant crops occur naturally are needed for risk assessment of transgenic crops [9, 12, 43–46]. Whether transgenes stably expressed in crop-wild hybrids [47] allow them to compete favorably [41, 48–51], persist, and spread more rapidly depends critically on the fitness advantage of the introduced resistance trait. Although wild mustard increased fitness overall when protected from the Bt susceptible subset of its herbivores [46], Bt-protected *B. rapa* was the lesser competitive Brassicaceae in nonagricultural areas with established vegetation. Both the overall fitness advantage of *B. rapa* plants protected with Bt *endotoxins*, and the weaker response in established vegetation are similar and complementary to the results of Stewart et al. [52] comparing transgenic Bt-*Brassica napus* and nontransgenic *B. napus* subjected to a defoliation episode that resulted in a 40%–60% decrease in percent herbivory, through the addition of larval diamondback moth in cultivated and natural vegetation plots. The similar increase in lifetime fitness in wild radish (*R. sativus*) with Bt sprays versus larval additions was more robust to habitat type differences than was *B. rapa*, suggesting that testing for fitness effects on wild relatives should involve representatives from more than one species under different field conditions.

Seed output of wild mustard that survived to maturity was not consistently affected by differential herbivory, with a significant increase in Bt-protected plants only in the last year of our study [46]. Therefore, our results and those of Stewart et al. [52] reflect lifetime fitness of *B. rapa*, including early mortality. This distinction may explain a lack of fecundity consequences from herbivory in some other potted plant studies of wild relatives of cole crops, which have shown strong compensation in terms of seed set ([53, 54] for *R. raphanistrum* and [55] for *B. nigra*). An average plant cover of ~90% grasses and forbs already established or building in the first months after germination of our test plants

contrasted starkly with less crowded growing conditions in a majority of previous studies testing for tolerance to herbivory in pots (e.g., [20, 56–59]) or weeded field sites (e.g., [60, 61]). These studies, and the small scale field study of Strauss et al. [62] on wild radish are not inconsistent with our fitness results if plants in these protected conditions rarely suffered mortality or if seed output was calculated only from plants that lived to reproduce (rather than including zero seed production for plants that died as juveniles). Blatt et al. [63] suggested that an increase in stress through multiple attacks by herbivores, simultaneous damage by pathogens, or poor growing conditions may curb tolerance to herbivory and allow herbivores to exert measurable fitness effects on plants. In our study, early mortality resulting in zero seed set and aborted flowers resulting in plants with few seeds were important factors in explaining the overall very strong effects of herbivory (or release from herbivory) on average lifetime fitness per plant. The subset of those plants that survived to set seed in our experiments showed a less dramatic reduction in seed output owing to higher herbivory, which was only a marginally significant difference from protected plants. Surviving plants with higher herbivory produced 25% less seed than protected plants, compared to over 50% less when juvenile mortality was included. Clearly, the impact of Bt transgenes on mortality and fecundity of wild crop relatives is complicated by what kind of herbivory and how much herbivory the plant would have normally sustained, the developmental stage of the plant, plant species, and the surrounding habitat conditions. However, the potential for increased fecundity and rates of spread were upheld in field experiments under natural field conditions, whether using naturally occurring wild relatives with herbivore manipulations or transgenic Bt plants.

Researchers and regulators involved in risk assessment face data gaps in predicting the consequences of host plant IR transgenes in wild plant populations [9, 14, 43, 44]. In the process of constructing data sets to aid in these assessments, we expect limitations in scientific quality because demographic data on the size and rate of spread of plant populations derived from transgenic crop-wild hybrids cannot be collected without increasing the likelihood of transgene escape in the process. Containment restricts effective experimental designs with transgenic hybrids because they are conducted in cages, greenhouses, or at locations where the taxon does not occur, and so cannot form hybrids. In each of these cases, relevant biotic or abiotic factors are likely to be lacking. On the other hand, simulation experiments with nontransgenic plants are only as predictive as the simulation is accurate, at least to the degree necessary to represent relevant factors. Nevertheless, several experimental tests involving unintended effects of Bt insecticidal traits on plant fitness, and all with different experimental approaches, have come to similar conclusions about plant fitness effects of herbivore resistance traits. In addition to those mentioned previously, Snow et al. [44] found that significant reductions in seed predation by gelechiids and tortricid pests occurred when wild sunflower expressed Bt toxin; these insecticidal plants tended to have higher numbers of undamaged seeds

per plant, in part because of producing significantly more mature flowers.

We consider decreased female fitness due to naturally occurring levels of herbivory to be realistic outcomes for California agricultural and grassland habitats. Whereas individual fitness should be closely linked to seed production, the ecological population consequences of producing more seeds depend critically on whether those seeds will establish. Based on our seed addition experiments, an increase in seed production can allow more rapid spread of wild radish in local habitats. Results of our microcosm experiments are consistent with our casual observations of wild radish persisting in experimental plots that previously had no radish plants. Wild mustard, on the other hand, is not likely to invade grasslands by producing more seed but can increase numbers in disturbed sites such as agricultural fields.

Tiered frameworks for risk assessment (e.g., [64]) are designed to reach an informed prediction of the likelihood that harm will occur from an action, using the minimal amount of data. Using the framework proposed by Raybould and Cooper [45], tests for a fitness advantage associated with Bt-based resistance in wild plants constitute a 2nd tier hazard assessment. One advantage of herbivore exclusion tests at this step in a risk assessment process, designed to determine the likelihood of increased weediness in crop-wild hybrids with introgressed plant defense transgenes, is that the tests can be carried out in the regions and habitats where hybrids are likely to form. Another is that transgenic constructs of different wild relatives are not necessary. However, as with our study on wild mustard, factors such as hybrid fertility and vigor, hybrid seed emergence, differences between transgene expression and topical sprays (issues from genetic load to toxicity range on herbivores), and the competitiveness of the F1 and backcross generations (e.g., [48–51]) can partially mitigate or exacerbate any undesirable outcome of host plant resistance traits [45]. Yet assessing the potential for increased weediness of plant species with novel traits in different, realistic habitats is critical, given the range of potential economic and environmental consequences [65, 66]. Therefore, herbivore exclusion experiments are practical tools for predicting potential effects of a range of insect resistance traits on the population dynamics of crop relatives that may receive these traits through gene flow from transgenic crops.

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