

The responses of crop – wild *Brassica* hybrids to simulated herbivory and interspecific competition: Implications for transgene introgression

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Brassica rapa grows as a wild and weedy species throughout the world and is the most likely recipient of transgenes from GM oilseed rape. For transgene introgression to occur, the critical step which must be realized, is the formation of an F₁ hybrid. Concerns exist that hybrid populations could be more vigorous and competitive compared to the parental species. This study examines the effect of simulated herbivory and interspecific competition on the vegetative and reproductive performance of non-transgenic F₁ hybrids and their parental lines. Several vegetative and reproductive performance measures were used to determine the effect of simulated herbivory and competition on the *Brassica* lines, including leaf length and biomass for herbivory and seedling height and biomass for competition. For defoliation experiments, *B. rapa* showed little response in terms of leaf length but *B. napus* and the F₁ hybrid responded negatively. *Brassica rapa* showed elevated biomass responses, but *B. napus* and the hybrid demonstrated negative responses to defoliation. Defoliation at the cotyledon stage had a slight effect upon final biomass with the F₁ hybrid performing significantly worse than *B. napus*, although seed counts were not significantly different. For the series of competition experiments, hybrids seemed to be more similar to *B. rapa* in terms of early seedling growth and reproductive measures. The underperformance of hybrid plants when challenged by herbivory and competition, could potentially decrease survivorship and explain the rarity of hybrids in field surveys. However, should transgene introgression occur, the dynamics of hybrids could change radically thus increasing the risk of gene flow from a transgenic oilseed rape crop to the wild recipient.

Keywords: *Brassica rapa* / *Brassica napus* / defoliation / *Brassica* hybrid / risk assessment

INTRODUCTION

Oilseed rape, *Brassica napus* L. currently occupies over 24 million hectares globally and often grows in a sympatric association with its wild relatives *e.g.* *Brassica rapa* L. and *Brassica oleracea* L. (Davenport et al., 2000). As a result, there is an intrinsic risk of hybridization and transgene introgression from genetically modified (GM) oilseed rape crops into naturalized or weedy *Brassica* species (Scheffler and Dale, 1994). It has been demonstrated that the most likely introgression recipient is *B. rapa* (Jørgensen and Andersen, 1994; Raybould and Gray, 1994; Warwick et al., 2003) which can grow as a weed in oilseed rape fields (Jørgensen et al., 1996) and naturalized in riparian ecosystems (Wilkinson et al.,

2003a). Numerous studies have indicated a high potential for hybridization between *B. napus* and *B. rapa* (Halfhill et al., 2004; Hansen et al., 2001; Jørgensen and Andersen, 1994; Landbo et al., 1996), which suggests that the risk of gene flow is high.

Bacillus thuringiensis (*Bt*) endotoxin-encoding genes have already been inserted into a number of experimental lines of oilseed rape (Halfhill et al., 2002; Raldugina et al., 2000; Stewart et al., 1996), but to date no commercial insect-resistant varieties have been made available. However in the future, both existing and novel *Bt* transgenes could be engineered into oilseed rape to manage many of the important insect pest species on the

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crop. Therefore, introgressed *Bt* transgenes could enhance the reproductive fitness of the recipient *Brassica* species (Stewart et al., 1997). The knock-on effects of this could be an enhanced weediness in the case of weedy *B. rapa*, an increase in its competitiveness over neighboring wild plants or an improved exploitation of new niche ranges in wild *B. rapa* (Weis, 2005). Theoretically, in a worst case scenario, these transgenic phenotypes with enhanced fitness could increase in number with possible undesirable effects on ecosystem functioning (Jørgensen et al., 1999). To determine whether this might be the case, we must first identify the key factors that regulate plant population size and, especially, the major causes of mortality in those wild species (Raybould et al., 1999).

In the past, gene flow risk assessment concentrated solely on the likelihood of hybridization without investigating the ecological consequences (Poppy, 2004). To determine ecological risk, we have proposed that an “exposure tree” approach is used (Sutherland and Poppy, 2005) which states that transgene introgression can only occur if all steps in the exposure tree are realized. This must always begin with the formation of an F_1 hybrid and subsequent backcrosses of these plants with the wild recipient before the gene can stabilize in the population (Wilkinson et al., 2003b). If any of the steps in the pathway are blocked, transgene introgression will not occur. Thus, the F_1 hybrid is the critical step in determining the size of the risk, as it will determine the number of transgenes which will ultimately be expressed in the wild relative (Linder and Schmitt, 1994; 1995). Therefore, understanding the dynamics of hybrid plants in response to selection pressures such as herbivory and competition is essential, especially if we are to infer what may happen if herbivory pressure is diminished in the presence of a fitness-enhancing gene such as *Bt*. The first logical step in assessing risk is to evaluate the performance of non-transgenic hybrid plants alongside the parental plants, under a range of differing selection pressures to determine whether compensation occurs in the hybrid which could give it a selective advantage over the parental types. By testing this system first with non-transgenic plants will provide us with essential baseline data which will allow us to develop a comprehensive risk assessment protocol for transgenic oilseed rape, should widespread cultivation occur in the future.

In the current work, several experiments were performed to investigate the effect of selection pressures on the performance of several non-transgenic *Brassica* lines, comparing oilseed rape \times naturalized *B. rapa* F_1 hybrids with the two parental lines. To assess this we used both vegetative and reproductive measures of plant

performance in response to varying levels of simulated herbivory and competition. Previous experiments focusing on consequences of gene flow have examined reproductive performance exclusively, concentrating on the genetic rather than ecological drivers in the system. However, populations cannot be studied without taking into account both their ecology and genetics (Lewontin, 2004) and it is vital to investigate the often-ignored parameters such as early growth responses. In this work we particularly concentrated upon early growth as we view this as the critical period in the plant’s growth, both in terms of herbivory and competition. In many respects, these vegetative indices may be more important than reproductive performance in determining how hybrid individuals fare in competition with seedlings of the parental species (Gressel, 2002) and modeling has suggested this (Rees and Long, 1992). Together, these experiments characterize comprehensively the effect of several selection pressures, on the early performance of F_1 hybrids between oilseed rape and wild *B. rapa* making direct comparisons with the parental types.

RESULTS

Ploidy determination

All three *Brassica* lines had their ploidy determined by flow cytometry. The parental types, *B. rapa* and *B. napus* were confirmed as being diploid ($2n = 20$, AA genome) and tetraploid ($2n = 38$, AACC genomes) respectively. The hybrid plants demonstrated a triploid level of ploidy ($2n = 3 \times = 29$, AAC genomes) and therefore their hybrid status could be confirmed. F_1 hybrids carry haploid sets of the A genome from *B. rapa* and AC genomes from *B. napus* making them triploid individuals.

Simulated herbivory

Early growth measurements (defoliation)

F_1 hybrids demonstrated a consistently smaller leaf length than *B. napus* and *B. rapa* at all three sampling dates after simulated defoliation (Tab. 1). These differences were most noticeable at the highest levels of defoliation at all three dates. This suggests that the hybrid is less well able to tolerate herbivory than its parental types.

Seedling biomass was similarly affected by defoliation in all three lines. Both parental types had a greater biomass than F_1 hybrids at 7 days, but the *B. rapa* was significantly greater than the other lines at both 14 and 21 days (Tab. 1).

Table 1. Leaf length and seedling biomass of *Brassica napus*, F₁ hybrid and *B. rapa* plants at 7, 14 and 21 d after 25, 50, 75 and 100% defoliation. Values are means (standard error in parentheses). *P* values are based on ANOVA and means with different superscript letters are significantly different (*P* < 0.05), according to the Tukey-Kramer test.

	<i>B. napus</i>												<i>B. rapa</i>				<i>F</i> -ratio	<i>P</i> value				
	F ₁ hybrid																					
	Treatment (level of defoliation)																					
Days after defoliation	Control	25%	50%	75%	100%	Control	25%	50%	75%	100%	Control	25%	50%	75%	100%	Control	25%	50%	75%	100%		
Leaf length (mm)	7	32.7 ^a (1.3)	32.5 ^a (1.3)	32.5 ^a (1.1)	29.9 ^a (2.5)	25.0 ^{ab} (0.9)	29.5 ^a (1.8)	24.6 ^{ab} (1.7)	25.9 ^{ab} (2.0)	20.5 ^{bc} (1.8)	15.3 ^c (1.4)	28.9 ^a (2.0)	31.8 ^a (1.1)	28.9 ^a (1.4)	31.4 ^a (2.2)	27.3 ^{ab} (1.5)	11.29	< 0.0001				
	14	52.1 ^a (1.7)	53.4 ^a (2.1)	47.1 ^{abc} (1.7)	44.3 ^{abc} (1.8)	39.1 ^c (1.9)	43.1 ^{abc} (2.3)	37.1 ^{cd} (2.2)	41.9 ^{abc} (2.2)	29.9 ^{de} (1.7)	25.3 ^e (1.7)	45.1 ^{abc} (3.1)	50.9 ^{ab} (2.7)	46.0 ^{abc} (3.2)	52.6 ^{ab} (3.3)	42.3 ^{abc} (2.8)	14.20	< 0.0001				
	21	87.5 ^a (1.6)	84.8 ^a (3.3)	80.0 ^a (3.7)	71.3 ^{abc} (2.1)	61.1 ^{bcd} (2.4)	57.8 ^{cd} (1.4)	53.6 ^{de} (2.1)	58.9 ^{cd} (2.4)	44.6 ^{ef} (1.6)	41.1 ^f (2.9)	84.4 ^a (3.8)	84.1 ^a (5.2)	78.2 ^{ab} (5.9)	84.4 ^a (4.1)	75.8 ^{ab} (4.0)	25.79	< 0.0001				
Seedling biomass (mg)	7	63.6 ^a (8.2)	58.5 ^a (5.9)	40.6 ^{abc} (1.6)	31.2 ^{bcd} (4.0)	36.1 ^{abcd} (3.2)	58.8 ^a (3.8)	39.3 ^{abcd} (6.1)	34.8 ^{bcd} (5.4)	21.6 ^d (2.3)	24.7 ^d (4.9)	67.9 ^a (9.1)	56.2 ^{ab} (6.7)	40.2 ^{abc} (2.6)	41.2 ^{abc} (2.5)	28.0 ^{cd} (3.2)	8.21	< 0.0001				
	14	148.1 ^{bc} (27.1)	285.9 ^{ab} (32.6)	96.1 ^{cd} (6.1)	158.0 ^{bc} (11.1)	80.7 ^d (10.8)	184.8 ^{ab} (15.6)	171.3 ^{bc} (27.2)	102.9 ^c (16.3)	76.9 ^d (14.5)	34.5 ^e (4.7)	219.5 ^{ab} (25.5)	344.8 ^a (46.2)	339.7 ^a (23.9)	176.0 ^{bc} (16.1)	166.3 ^{bc} (21.4)	25.00	< 0.0001				
	21	240.9 ^{def} (30.9)	613.0 ^{ab} (80.7)	326.0 ^{cd} (63.4)	646.1 ^{ab} (77.2)	173.4 ^f (36.5)	322.2 ^{cde} (42.6)	188.2 ^{ef} (12.8)	150.3 ^f (21.9)	142.5 ^f (15.4)	249.7 ^{def} (40.6)	512.8 ^{abc} (44.2)	549.6 ^{abc} (57.7)	679.2 ^a (65.8)	523.6 ^{abc} (50.3)	360.3 ^{bcd} (27.6)	21.10	< 0.0001				

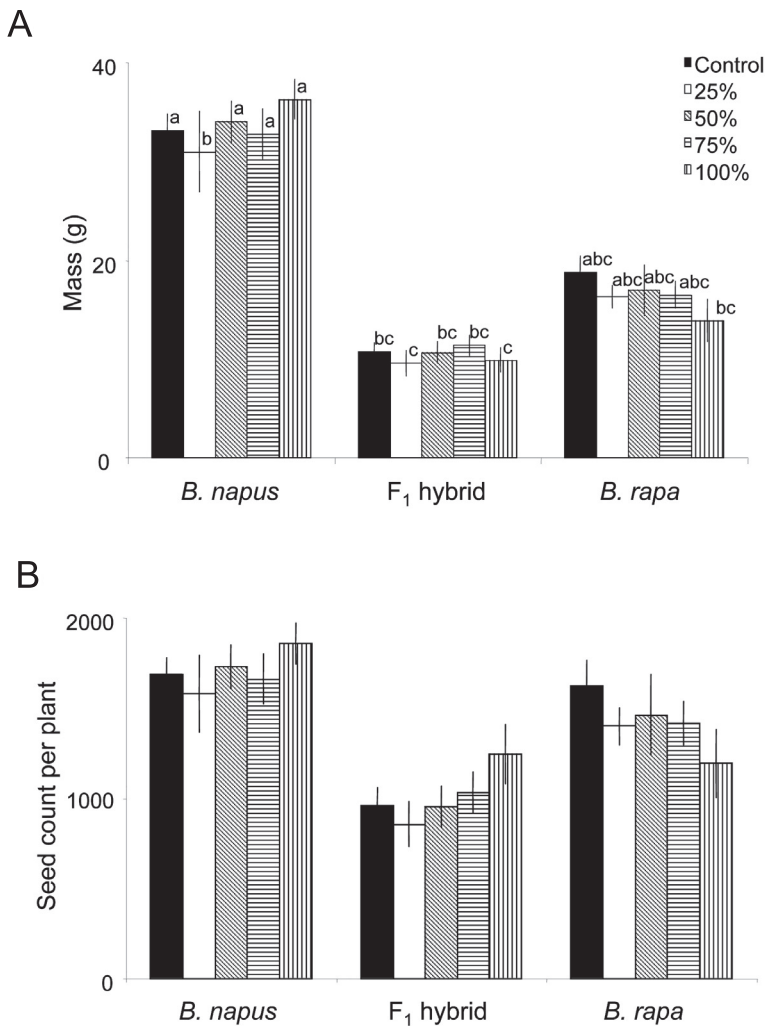


Figure 1. Single plant dry biomass (A) and seed count per plant (B) of *Brassica napus*, F₁ hybrid and *B. rapa* plants in response to 25, 50, 75 and 100% defoliation. Different letters above the means indicate significantly different means ($P < 0.05$), according to the Tukey-Kramer test. Bars represent standard errors.

Final growth measurements (defoliation)

Brassica napus had a greater dry weight at senescence than the F₁ hybrid ($F_{14,150} = 9.0, P < 0.0001$), although there were no significant differences between the hybrid and *B. rapa* (Fig. 1A). However, a subsequent loss in reproductive fitness in the hybrid compared to the parents was not borne out by the seed counts ($F_{14,150} = 1.5, P = 0.13$) (Fig. 1B). This is perhaps not unsurprising, given that we would expect a single defoliation event at the cotyledon stage to have little or no effect upon final biomass and seed count.

Interspecific competition

Early growth measurements (competition)

Seedling height was used a growth proxy measurement. In terms of height, the F₁ hybrid plants were more similar to *B. rapa* than to its other parental line. *B. napus* had a consistently taller seedling height than *B. rapa* and the F₁ hybrid (Tab. 2). With regard to the interspecific competition, we found that there was no significant impact upon seedling height on all three sampling dates (Tab. 2). Similarly, seedling biomass was not significantly affected

Herbivory and competition responses of *Brassica* hybrids

Table 2. Seedling height and biomass of *Brassica napus*, F₁ hybrid and *B. rapa* after competition with 5, 10 and 15 wheat plants (W) at 7, 14 and 21 d after emergence. Values are means (standard error in parentheses). *P* values are based on ANOVA and means with different superscript letters are significantly different (*P* < 0.05), according to the Tukey-Kramer test.

		<i>B. napus</i>				F ₁ hybrid				<i>B. rapa</i>				<i>F</i> -ratio	<i>P</i> value
		Treatment (level of competition)													
		Days after emergence													
		Control	5W	10W	15W	Control	5W	10W	15W	Control	5W	10W	15W		
Seedling height (mm)	7	34.7 ^a (3.2)	41.0 ^a (1.0)	38.3 ^a (1.1)	37.1 ^a (1.6)	15.0 ^b (1.6)	12.0 ^b (0.8)	13.1 ^b (1.5)	13.7 ^b (0.8)	17.0 ^b (0.9)	12.8 ^b (0.9)	17.2 ^b (1.5)	14.0 ^b (1.1)	39.88	< 0.0001
	14	84.9 ^a (5.9)	102.9 ^a (4.7)	91.5 ^a (4.9)	91.0 ^a (3.7)	50.4 ^{bc} (3.6)	44.1 ^{bc} (2.5)	58.3 ^b (4.3)	50.3 ^{bc} (3.3)	52.5 ^{bc} (3.0)	39.3 ^c (1.8)	49.2 ^{bc} (3.1)	45.3 ^{bc} (2.5)	29.73	< 0.0001
	21	173.5 ^{ab} (5.8)	196.7 ^a (7.1)	184.5 ^a (10.8)	186.7 ^a (10.5)	137.0 ^{bc} (11.1)	124.4 ^c (10.8)	122.6 ^c (7.5)	105.7 ^c (10.7)	120.7 ^c (4.8)	99.1 ^c (6.6)	112.1 ^c (5.6)	103.2 ^c (7.1)	15.32	< 0.0001
Seedling biomass (mg)	7	8.5 ^a (0.1)	7.3 ^{ab} (0.3)	5.9 ^b (0.2)	7.1 ^{ab} (0.5)	1.9 ^{cd} (0.1)	1.9 ^{cd} (0.1)	1.5 ^d (0.1)	1.9 ^{cd} (0.1)	2.2 ^c (0.1)	2.1 ^c (0.1)	1.8 ^{cd} (0.1)	2.3 ^c (0.3)	115.97	< 0.0001
	14	14.3 ^a (0.3)	13.4 ^a (0.7)	13.9 ^a (0.6)	14.1 ^a (0.6)	3.3 ^c (0.1)	3.5 ^c (0.2)	5.4 ^b (0.7)	3.8 ^c (0.2)	5.6 ^b (0.2)	5.8 ^b (0.4)	6.0 ^b (0.3)	5.3 ^b (0.2)	92.26	< 0.0001
	21	26.3 ^a (0.5)	26.5 ^a (2.4)	19.2 ^b (1.3)	25.1 ^a (3.6)	6.4 ^{cd} (0.2)	6.3 ^d (0.7)	6.7 ^{cd} (0.9)	4.5 ^d (0.4)	13.6 ^{bc} (0.9)	9.4 ^c (0.3)	8.6 ^{cd} (0.8)	7.7 ^{cd} (0.9)	50.68	< 0.0001

by interspecific competition in all three *Brassica* lines studied. Clearly, *B. napus* exhibited more growth than both *B. rapa* and F₁ hybrids during the trial, and *B. rapa* also performed better than the hybrids.

Final growth measurements (competition)

Overall in competition experiments, *B. napus* appeared to have a greater dry weight at senescence than both *B. rapa* and the F₁ hybrid, and in turn the hybrid was greater than *B. rapa* ($F_{11,120} = 7.1$, $P < 0.0001$) (Fig. 2A), although with regard to the treatments, competition only slightly affected final biomass in the three *Brassica* lines studied, and not surprisingly, this was most noticeable in the highest competition treatment (15 wheat plants), although all competition treatments did not differ significantly from each other. Furthermore, and possibly more significantly in terms of reproductive fitness, *B. napus* plants produced a significantly greater number of seeds than *B. rapa* and the F₁ hybrid ($F_{11,120} = 5.8$, $P < 0.0001$) at the highest levels of competition (Fig. 2B). Whilst *B. rapa* had a slightly lower dry weight than the hybrid (Fig. 2A), they

actually produced a similar number of seeds. The high variability in samples, especially for the hybrid meant that there were few significant differences.

DISCUSSION

This work provides clear evidence that F₁ hybrids between *B. napus* and wild *B. rapa* clearly have a lower vegetative “performance” than the two parents when compromised by the two selection pressures investigated; simulated herbivory and interspecific competition. This closely mirrors the findings in similar F₁ hybrids between *Raphanus raphanistrum* and *B. napus* (Guéritaine et al., 2003).

Simulated herbivory

F₁ hybrid plants consistently demonstrated a negative response to simulated herbivory at the seedling stage, meaning a loss in vegetative performance which in a “natural” situation in the presence of competitors could decrease survivorship. The fact that the hybrid is most

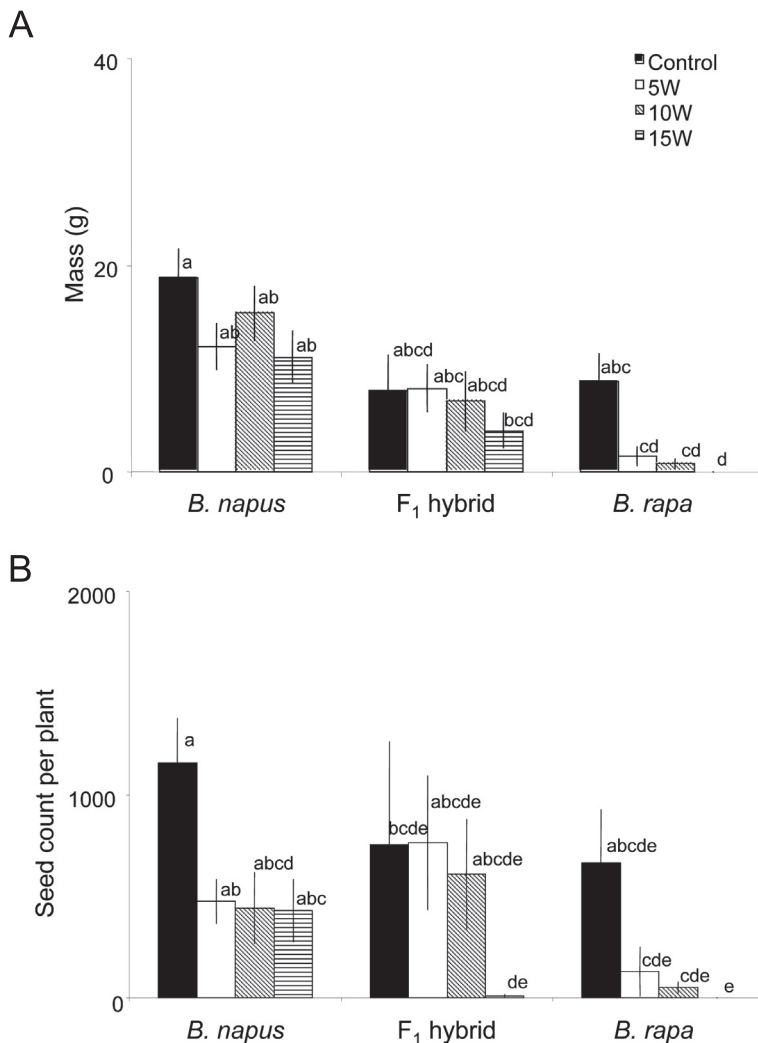


Figure 2. Single plant dry biomass (A) and seed count per plant (B) of *Brassica napus*, F₁ hybrid plants and *B. rapa* in response to competition with 5, 10, 15 wheat seedlings (W). Different letters above the means indicate significantly different means ($P < 0.05$), according to the Tukey-Kramer test. Bars represent standard errors.

affected by simulated herbivory is an important finding. This may also go some way to explaining why *B. napus* × *B. rapa* hybrids, when quantified in sympatric sites in the UK, were found to be relatively rare (Scott and Wilkinson, 1998). Overall, *B. rapa* appeared to compensate more for mechanical damage than the commercial line (*B. napus*), which in turn fared better than the F₁ hybrid. However, this is not unusual, as wild counterparts are often more fit than their domesticated lines, e.g. Gavloski and Lamb (2000b) found that *Sinapis alba* also compensated more for simulated damage than *B. napus*.

Tolerance by plants to herbivory is a well-known phenomenon which has been demonstrated in a number of plant-animal systems (Strauss and Agrawal, 1999; Trumble et al., 1993). The actual endogenous mechanisms behind tolerance are poorly understood but are believed

to be a complex of increased photosynthesis, higher relative growth rate and the ability to shunt carbon stores from roots to shoots after damage (Trumble et al., 1993). This was demonstrated in the work herein by increases in biomass by the two parental types but not in the hybrid.

One further point of interest is the compensation which appears to occur at low levels of defoliation. This stimulatory effect, known as hormesis, is often seen at low levels of a wide range of environmental stressors (Davis and Svendsgaard, 1990). The reason for hormesis is not completely understood, but it is conjectured that low level exposure to a stressor may trigger a hyper-response by the organism.

Our experiments also demonstrated that the response to a single defoliation event tended to be relatively ephemeral and many of the plants, including the F₁

hybrids, recovered from total cotyledon loss after three weeks. It is known that most plant species tend to compensate more for single defoliation events (Cartwright and Kok, 1990). However, observations that plants compensate very little for damage just after germination (Bardner and Fletcher, 1974) have been disproved in this particular system. In fact, Mauricio et al. (1993) suggested that cotyledons and the first true leaves might actually be more valuable to the plant, as they have a significantly higher photosynthetic rate than older leaves.

In undertaking these investigations we were also fully aware that simulated herbivory is often regarded as a poor substitute for actual herbivory (Agrawal, 1998; Karban and Baldwin, 1997) because simulated and natural herbivory evoke different responses in plants in terms of induction of phytochemicals. However, a very similar study (Gavloski and Lamb, 2000a) reported that the compensation responses to artificial herbivory were indistinguishable from actual herbivory, so we felt justified in using simulated herbivory in this instance. It also meant that we could ensure a high degree of accuracy in the level of defoliation applied, compared to the high variability expected had insects been used.

Interspecific competition

Again the hybrid plants typically had a lower vegetative performance than the two parents when compromised by interspecific competition. This was shown by two growth proxy measurements - seedling height and biomass. These vegetative parameters are important in determining how hybrid individuals fare in competition with other plant species (Gressel, 2002). Overall, during early growth, the hybrid seemed to behave more like *B. rapa* than the *B. napus*, with the commercial line performing best. This is the converse to our findings with *Brassica* lines and their hybrids in the USA in which *B. rapa* was the best competitor (Halfhill et al., 2005). In this instance, the *B. rapa* used was a weedy biotype, so we would expect the weedy species to be the better competitor.

Whilst one would predict the poor early vegetative performance of the F₁ hybrid to translate to a loss in later vegetative performance, this was not borne out by the present work, in which the hybrid had a greater plant mass at senescence than *B. rapa*, albeit significantly less than *B. napus*. However, hybrids did not produce any more seed than *B. rapa*. The fact that the hybrid exhibits no competitive advantage over *B. rapa* is an important finding, given that we have already demonstrated that hybrids are significantly affected by simulated herbivory.

Furthermore, in an analysis of F₁ seeds, we found only 15% normal seeds, 73% aborted seeds, and 12% with precocious germination. This had previously been reported by Hauser and Østergård (1999). Again, all of these factors may be an explanation as to why hybrid numbers are low in the wild (Scott and Wilkinson, 1998).

Hybrid fitness

The concern with the widespread introduction of GM *Brassica* crops is that hybridization with wild species could in theory produce genotypes which could have a greater fitness than their parents. However, many have concluded that natural hybridization is of little evolutionary importance because hybrids, in general, are less fit than their progenitors (Arnold and Hodges, 1995), as we have found in this instance. However, recent studies have found that the fitness of hybrids is by no means uniform, but rather contain several genotypic classes possessing lower, equivalent or higher levels of fitness relative to their parents (Arnold and Hodges, 1995). *Raphanus sativus* hybrid fitness was greater than its weedy parents (Klinger and Ellstrand, 1994). Similarly, and of direct relevance to this study, Hauser et al. (1998a) found that weedy *B. rapa* × *B. napus* F₁ hybrids had an intermediate fitness compared to their parents, and were significantly more fit than *B. rapa*, which is surprising given the data herein. A possible reason for this is that the Danish weedy *B. rapa* populations were more closely related to *B. napus* than our naturalized *B. rapa* population, which has presumably been growing in relative genetic isolation for several hundred years. In a follow up study in the Danish *B. rapa*, it was found that the F₂ hybrids did have a much-reduced fitness (Hauser et al., 1998b), indicating a further possible bottleneck for gene flow from GM oilseed rape.

Implications for transgene introgression

In a semi-natural habitat, a small number of hybrids would typically exist surrounded by a greater number of the wild relative, and it is likely that the hybrid would be most affected by herbivory, given the results herein. If the hybrids are largely released from herbivore pressure, by the presence of a *Bt* transgene, then their fitness and numbers could increase. Thus, there could be selection for the hybrids containing the transgene, and thus potential for more gene flow into wild plants, irrespective of dynamics of the wild plants, simply because more hybrids containing the transgene will be available to backcross into the wild relative. As a result, unless there is a

significant trade-off or cost of expressing the transgene, the gene could slowly increase in frequency in the wild plants. This may not have any ecological significance if the dynamics of these wild plants is not affected by herbivory and may only matter from a biodiversity or conservation perspective if there is a rare lepidopteran species feeding on the plants whose populations could be directly affected by consuming the *Bt* toxins. The next important step will be to evaluate whether the addition of this insect resistance transgene in a hybrid plant does actually enhance the fitness of the hybrid plants, although early indications tend to suggest that this will be unlikely (Halfhill et al., 2005).

Studies such as this can serve to identify short-term dynamic effects, but may not be able to determine the significance of these changes over longer timeframes; *i.e.* if a significant change is reported in a “snapshot” study such as this, this may be not significant over a longer timeframe when more complex ecological dynamics contribute variation into the system. Conversely, a small effect which means little in a single generation may accumulate into a more profound effect further along the “pathway”. The generation of accurate models, such as that used by Watkinson et al. (2000), which are based on empirical data from investigations such as this, will be vital to determine the longer-term ecological implications of the increased presence of hybrids in the environment. This is especially the case when we want to consider changes in ecological stressors such as competition and herbivory and will ultimately provide some indication of the likelihood and rate of spread of transgenes in the environment.

MATERIALS AND METHODS

Plant material

B. rapa seeds were originally obtained in July 2001 from a single naturalized riparian population growing on the Kennet and Avon Canal, Claverton, Bath and NE Somerset, UK. A commercial variety of winter oilseed rape, *B. napus* cv. Apex (Advanta Seeds UK Ltd, Norfolk, UK) was also used. F₁ hybrids were created between *B. napus* and *B. rapa* in the glasshouses at the Universities of Southampton and Reading by emasculation and cross-pollination (Downey et al., 1980) with *B. napus* as the male donor and *B. rapa* as the recipient. Multiple crosses were made between approximately 20 individuals of the two parents at the two sites and seed material bulked. *B. rapa* is an obligate outcrosser due to the presence of self-incompatible genes; as a result, little or no seed is set

when *B. rapa* is self-pollinated. Flower buds were emasculated with superfine No. 4 forceps and anthers from the donor brushed against the recipient stigma. Emasculated flowers were bagged with pollination bags (PBS International, Scarborough, UK) for 48 h to prevent contamination with other pollen grains.

Ploidy determination

The hybrid status of individuals was confirmed by flow cytometry. Fresh leaf samples were sent to Plant Cytometry Services (Schijndel, The Netherlands) for flow cytometry analysis. Each series of leaf samples included relevant positive controls of *B. napus* cv. Apex and *B. rapa* (from the natural population on the Kennet and Avon Canal, Bath, UK). All experimental samples were coded before dispatch. Immediately prior to flow cytometry, leaves were cut in ice-cold neutral buffer containing 300 mM sucrose, 80 mM KCl, 20 mM NaCl, 15 mM HEPES, 1 mM EDTA, 0.5 mM Spermine, 0.2% (v/v) Triton X-100 and 2 mg.L⁻¹ DAPI. Nuclei were isolated from the tissue suspension by filtering through a nylon filter with a 40 µm mesh. The stained suspension of nuclei was passed through a PAS II flow cytometer (Partec, Munster, Germany) using the excitation filters UG-1, BG-38, KG-1 and TK-420 and the emission filters TK 560 and GG 435. DNA histograms were produced using FLOWS 2.00 software (Partec, Munster, Germany). Crude confidence intervals were calculated for the position of the 2C and 4C peaks according to Rabinovitch (1994).

Simulated herbivory

Simulated herbivory was designed to mimic herbivory by diamondback moth (DBM), *Plutella xylostella* (L.), considered to be the most serious insect pest of *Brassica* species (Talekar and Shelton, 1993). This was a “concentrated” type of defoliation in which large areas of leaf were removed, regarded as being similar in nature to the damage caused by DBM. The economic threshold for DBM in oilseed rape is approximately 25 to 30% damage at the cotyledon stage (Talekar and Shelton, 1993), which served as a guideline for experimental treatments.

Seeds of each of the three plant lines were germinated in Levington’s No. 2 compost in 5 cm plastic pots. All seedlings were kept in growth chambers (22 °C day [D]: 20 °C night [N], photoperiod 12D:12N, rH 55–60% rH) at all times. For each experiment, 40 seedlings of each of the three *Brassica* lines were defoliated once, at the cotyledon stage or growth stage 1.00 (Sylvester-Bradley et al., 1984) at 7 d after appearance at soil surface.

Percentage defoliation is most easily determined at this stage because the cotyledons are small and easily manipulated. There were four levels of simulated folivory (10 seedlings for each for each experiment); 25, 50, 75 and 100% of the cotyledon area was removed using microdissecting scissors with 25% damage having one cotyledon lobe removed, 50% having two lobes removed, 75% having three removed and 100% all lobes were excised. Control groups of ten seedlings of each of the three lines with no damage were also introduced for each experiment. All pots and seedlings were assigned randomly to the bench in the growth chamber. Several separate experiments were performed during 2003 and 2004 and are described below.

Early growth measurements (defoliation)

B. rapa, *B. napus* and F₁ hybrid seedlings were defoliated once at the cotyledon stage with four levels of defoliation. Leaf length assessment consisted of measuring the length of all true leaves (excluding the petiole), using a pair of electronic calipers (RS, Corby, Northants., UK) at 7, 14, and 21 d after defoliation (dad). The same groups of seedlings were used throughout the experiment. After recordings were taken, these plants were retained for an assessment of final biomass (see below).

Again in a separate trial, whole seedlings were removed from the soil and separated into leaves, roots and stems. Roots were washed in distilled water. Seedlings were placed in paper dishes and dried at 60 °C for 48 h then weighed to find the biomass. Sampling in this instance was destructive so this necessitated three groups of 50 seedlings for each of the dates after defoliation, although all were defoliated on the same date.

Final growth measurements (defoliation)

Seedlings from the leaf length measurement group of plants were vernalized in a cold room (4 °C day [D]: 4 °C night [N], photoperiod 10D:14N, rH 70–75% rH) for 74 d. This was essential to induce flowering in all three *Brassica* lines, as without this period all plants would have remained vegetative. After vernalization, plants were repotted in John Innes No. 2 compost in 3 L pots and transferred to a polythene-clad tunnel greenhouse (with ambient temperature), to protect the young plants from mammalian herbivory and then removed to an open grassed area within a walled garden at Chilworth Science Park, Southampton prior to growth stage 3.00 (Sylvester-Bradley et al., 1984) to allow pollinators to visit flowers. *B. rapa* is highly self-incompatible and therefore requires

insect and / or wind pollination. All plants were harvested for determination of aboveground biomass, dried at 60 °C for 48 h, and weighed. Seeds were extracted from individual plants *via* sieving and weighed. Seed count per plant was estimated by calculating the 1000 seed weight for each of the three lines using a mean of three samples.

Interspecific competition

Seeds of each of the plant lines were sown in Levington's No. 2 compost in 5 cm plastic pots. Competition was provided by winter wheat (*Triticum aestivum* cv. Malacca) and treatments were as follows: a single *B. napus* seedling and 0, 5, 10 and 15 wheat seedlings; single *B. rapa* seedling and 0, 5, 10 and 15 wheat seedlings and finally a single F₁ hybrid seedling and 0, 5, 10 and 15 wheat seedlings. There were 10 repetitions per treatment in each case. All seedlings were kept in a glasshouse (20 °C day [D]: 15 °C night [N], photoperiod 16D:8N, rH 55–60% rH) at all times. Pots were assigned randomly to benches in the glasshouse.

Early growth measurements (competition)

Seedling height was used as a growth proxy and plants were measured on 7, 14 and 21 d after germination. Height measurements were taken from the base of the hypocotyl to the tallest part of the seedling. Seedling biomass was recorded *via* a separate duplicate experiment as this necessitated destructive sampling. Biomass measures were taken of the whole intact seedling on 7, 14 and 21 d after germination in each treatment. Ten seedlings for each treatment were used. Seedlings were removed from the soil, washed with water, dried and placed in paper dishes and were then oven dried for 48 h at 60 °C and weighed individually.

Final growth measurements (competition)

Productivity of the three *Brassica* lines was analyzed in pot experiments at the University of Southampton in summer 2004 to investigate response to increasing level of competition. The vegetative dry weight and seed mass and thus seed count were determined to give an indication of reproductive fitness of the plants.

All plants were again vernalized in a cold room using the conditions as previously described. After vernalization, plants were repotted in John Innes No. 2 compost in 3 L pots and transferred to an external open gravel area (with ambient temperature) at the University of Southampton. Plants were arranged at random and spaced so as

to prevent additional competition. Plants were covered with a large plastic netting cage to protect the plants from herbivory but removed immediately prior to growth stage 3.00 (Sylvester-Bradley et al., 1984) to allow pollinators to visit flowers. All individual plants were harvested for determination of aboveground biomass, dried at 60 °C for 48 h, and weighed to determine final biomass of plants. Seeds were extracted *via* sieving and weighed. Again, seed count was estimated using the 1000 seed weight.

Statistical analyses

Data were analyzed using the generalized linear models procedure. All variables were tested for normality and homogeneity of variances. As a result, so as data were normalized, all data were transformed to natural logarithms, except for seed count data where it was more appropriate to use log₁₀ transformations. Tukey-Kramer Multiple Comparison tests were used to test for differences between treatments (Minitab release 13.1). Data for each of the three dates were analyzed in separate ANOVAs.

ACKNOWLEDGEMENTS

We thank Kate Tuner for providing some of the F₁ hybrid seed used in experiments. This was part of the BBSRC and NERC funded Gene Flow in Plants and Microorganisms Initiative Project. It was a consortium-based project with the University of Reading, National Industry for Agricultural Botany, Centre for Ecology and Hydrology, Horticulture Research International and the University of Bath.

Received January 5, 2006; accepted April 24, 2006.

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