Backcrosses to *Brassica napus* of hybrids between *B. juncea* and *B. napus* as a source of herbicide-resistant volunteer-like feral populations

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**A B S T R A C T**

Introgression between genetically modified (GM) crops and wild relatives is considered to potentially modify the genetic background of the wild species. The emergence of volunteer-like feral populations through backcross of hybrids to the crop is also a concern. The progeny of spontaneous hybrids between mutant herbicide-resistant oilseed rape (*Brassica napus*) and wild *B. juncea* was obtained. Parents, \(F_2\) and \(BC_1\) to *B. napus* were planted together in the field so as to study their performance. The chromosome number of \(BC_1\) followed a Normal distribution. Mendelian ratio of the herbicide-resistance gene was found. The \(F_2\) produced less seeds than *B. napus*, and \(BC_1\) had intermediate production. Herbicide-resistant \(BC_1\) were not different of their susceptible counterparts for plant weight, seed weight and seed number, but most of them exhibited *B. napus* morphology and larger flowers than the susceptible \(BC_1\). They displayed an important genetic variability allowing further adaptation and propagation of the herbicide-resistance gene. Pollen flow to susceptible plants within the mixed stand was observed. As a consequence, the resistant \(BC_1\) produced with *B. napus* pollen could frequently occur and easily establish as a false feral crop population within fields and along roadsides.

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1. Introduction

In the framework of current research on the risk assessment of the commercial release of genetically modified (GM) crops, one of main concerns is whether transgenes could persist in the agro-ecosystem [1]. In the case of oilseed rape (*Brassica napus*), transgenes could escape through volunteers (plants descended from seeds shattered before or spilled during harvest on previous years and that can survive for years into the soil seed bank) [2] and affect both the habitat [3] and the quality of crop harvest [4]. They could also escape via spontaneous hybridization with wild relatives and subsequent introgression [5,6]. Volunteers and hybrids were observed in the agro-ecosystem field together with sown crops and in their neighborhoods [7,8].

The introgression between a genetically modified crop and a wild relative is generally thought to potentially modify the genetic background of the wild species, which thus generates either concerns about its weedingness and competitiveness in the agro-ecosystem or the risk of its extinction [6,9,10]. The backcrossing of hybrids with the crop parents could also occur, which could result in the introgression of wild characteristics into feral and volunteer populations. This process is generally underestimated and little investigated because the crop type is considered to be unfit to adapt to wild habitats [11]. However, the resulting plants are thought to be too close to volunteers and to deserve separate studies.

Gene flow between *B. napus* and *B. juncea* has received less attention compared to the *B. napus/B. rapa* case [12]. *B. juncea*, a wild relative native from Central Asia, widely cultivated and naturalized worldwide, is frequently found as a weed and a ruderal component of roadsides and waste places. It is a tetraploid species like *B. napus* with which it shares the genome A. It is preferentially self-pollinated with considerable outcrossing rate. Hybrids and subsequent backcross generations between *B. napus* and wild *B. juncea* were successfully obtained by open pollination [13–15]. The relatively high compatibility between *B. napus* and wild *B. juncea* raises the biosafety concern of transgene introgression. Di et al. [16] found that the hybrids formed between *B. napus* and wild *B. juncea* revealed maternal effects, high vegetative fitness and seed dormancy, which could increase the probability of the survival of hybrids after the occurrence of gene flow. However, Wei and Darmency [17] showed that gene flow could be hampered by low seed size of hybrids because of the low emergence, low survival rate and delayed flowering due to smaller seeds. Therefore, it is not clear under which form the progeny of interspecific hybrids between these two species could survive and then transfer transgenes to further generations in the realistic agro-ecosystem where un-cropped and cropped areas coexist.
In this study, we aim to investigate the behavior of the progenies of spontaneous hybrids between wild *B. juncea* and a herbicide-resistant germplasm of *B. napus* similar to those commercialized cultivars under the Clearfield® technology. We studied the formation of F2 and BC1 progenies, whether they may grow and reproduce under field conditions, and whether their performances are changed according to the presence of the herbicide-resistance gene. Instead of exploring the possibility of transgene introgression into the wild species, which has been already explored [18], this work focused on the likelihood of the emergence of backcrossed plants that could behave like *B. napus* volunteer-like plants.

2. Materials and methods

2.1. Hybrids of *B. juncea* × *B. napus*

A wild accession of *Brassica juncea* (2n = 36, AABB) from Turkey was obtained from the INRA laboratory collection of weeds (ref. X04-021) in Dijon, France. A mutant *B. napus* cv. Brutor (2n = 38, AACC), bearing a single-gene dominant resistance to chlorsulfuron, a sulfonylurea herbicide inhibiting the AcetoLactate Synthase (ALS), was provided by Renard [19]. These two species were grown in mixture in insect-proof cages continuously provided with houseflies, *Musca domestica*, in order to facilitate random pollination. The seeds harvested from *B. juncea* were sown in a greenhouse at 22°C under natural light, and then two-leaved seedlings were sprayed with 2.5 g a.i. ha⁻¹ chlorsulfuron in 300 L of water (Glean, Du Pont de Nemours, France). Eleven out of 9238 seedlings survived (0.13%), and these plants were confirmed to be interspecific F1 hybrids since they showed molecular markers of both parents (data are not shown).

2.2. Backcrosses into *B. juncea* and *B. napus* parents

Branches of all F1, the hybrid plants were cut and planted in the soil to multiply the hybrids. The hybrid cuttings were grown in the greenhouse and entirely covered by a pollen-proof net. Anthers of flowers of 24 cuttings from all eleven hybrids were removed and the stigmas received pollens from wild *B. juncea* by hand to generate a BC1 generation (BC1J). On 22 other cuttings, emasculated flowers received pollens from a conventional herbicide-susceptible *B. napus* cv. Drakkar to obtain a BC1 generation (BC1N) segregating for herbicide resistance and susceptibility (although not yet identified at that stage). The rest of hybrid cuttings were kept isolated by a pollen-proof net and served to get a bulked F2 generation (Fig. 1). Pollen fertility was checked by staining pollens of 5 flowers of each of 6 F1 hybrids with Alexander solution [20]. Number of pollinated flowers, seed number, per-seed weight and germination rate were recorded. Five hundred F2 seeds, 100 BC1N and 50 BC1J seeds were sown in the greenhouse and sprayed with the herbicide Glean as described above, and then seedlings alive were counted.

2.3. Field trial

Seeds of conventional herbicide-susceptible *B. napus* cv. Drakkar, wild *B. juncea*, BC1J, BC1N, and F2 were sown in Giffy 7 pots (Jiffy Prod. Int., Norway) in the greenhouse in order to promote simultaneous germination, and then the seedlings were transplanted into the field in Dijon, France, on April 2007. No herbicide treatment was applied so that the resistant or susceptible phenotype of the hybrid progeny could not be identified at that stage. In one experiment, *B. juncea*, BC1J and F2 were randomly planted in a bare field. In another place, *B. napus*, *B. juncea*, BC1N and F2 were planted randomly in the field with four blocks. Each block included three rows 30 cm apart and 11 m long, with one plant every 0.6 m. The blocks were enclosed in an insect-proof net (12 m wide/15 m large/2 m high, 2 mm mesh) to prevent insect attack, and houseflies were continuously provided in order to facilitate random pollination. *B. juncea* plants flowered earlier than *B. napus* in our field experiment, although the overlapping period in most field conditions is long enough to allow spontaneous hybridization to occur, as showed in the cage experiment when we get the hybrids (and in [13–15]). Consequently, they were hardly comparable to the other plant types and were no more considered in the following study.

The *B. napus* versus *B. juncea* morphology (large versus small habit) was visually noted before flowering. The flower sizes of all BC1N and F2 plants were visually rated in two categories: as large as that of *B. napus* versus at least 2/3rd smaller. In order to estimate the DNA content of the plants, whenever possible, a piece of fresh leaf was sampled to perform flow cytometry as described by Akinerden [21] and estimate (the G1 peak) with *B. juncea* as the control. When the first pods were about to open, the surviving plants were harvested. The vegetative parts were separated from the pods and dried in an oven for 48 h at 80°C and weighted. Seed weight and seed number were measured. The weight of one thousand seeds was calculated and expressed as per-seed weight.

Up to 2000 seeds of every harvested plant were sown in the greenhouse and sprayed with chlorsulfuron as described above. In order to assign a phenotype to the BC1N and F2 plants, we considered that a resistant one would ideally produce 75% of resistant offspring if entirely self-fertilized, while a susceptible one would produce no resistant seedlings. Because of possible biased segregations after the interspecific hybridization and the backcrosses on the one hand, and because of some potential cross-pollination that could have occurred in the field on the other hand, we interpreted as susceptible all the plants showing a progeny containing less than 5% resistant seedlings, which could correspond to a maximum rate of fertilization by resistant pollen. Otherwise the plant was designated as resistant.

2.4. Statistical analysis

Mean values are given with their 95% confidence limits. Chi-square analysis was employed to test herbicide-resistant rate of BC1 and F2 with the expected Mendelian segregation ratios. Analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test were carried out to compare plant weight, seed weight, seed number, per-seed weight and germination rate of the plants in the field trial. Bartlett’s test was used for testing variance homogeneity and data were log-transformed to ensure a normal distribution. We followed a fixed ANOVA model including a
3. Results

3.1. Reproduction of the F1 hybrids

All the F1 hybrids are herbicide-resistant, but it is not known if some susceptible ones could have occurred because the initial screening identification used the herbicide-resistance trait. The F1 hybrids produced 34 ± 3% viable pollens. They produced fewer seeds per pollinated flower when backcrossed with B. juncea pollen than when backcrossed with B. napus pollen (5.9 and 12.4%, respectively). Self-pollinated hybrids produced seeds (Table 1). The backcross with B. napus pollen produced heavier seeds, and they germinated better than the seeds produced with B. juncea pollen ($\chi^2 = 7.2$, significant at $p < 0.01$).

3.2. Characteristics of the backcrosses

G1 peak values of flow cytometry results of BC1N plants were Normally distributed: N ($m = 101.3, \sigma = 8.7, p = 0.82$) (Fig. 2). The G1 peak values of BC1J plants also fitted a Normal law distribution, N ($m = 95.6, \sigma = 6.7, p = 0.36$), but the mean value was significantly lower than that of BC1N by 5.7 units ($t = 2.9, p = 0.007$), very close to that of the control B. juncea parent ($m = 93.7$).

Both direct seedling treatment and phenotype inference from progeny analysis of the plants grown in the field experiment showed expected Mendelian ratios: 3:1 resistant:susceptible in F2, and 1:1 for both BC1J and BC1N plants (Table 2). Some plants growing in the field and determined as susceptible produced at least one resistant descendant (Table 3): 13 BC1NS individuals, later on referenced BC1N*, showed 0.8 ± 0.6% resistance on average in their progeny (0.34% for all the BC1NS plants); the 4 F2S plants produced 3.6% resistant progeny; and 2 conventional herbicide-susceptible B. napus plants had 0.13% resistant descendants (0.007% for all the B. napus plants).

3.3. Growth and reproduction in the field

The experiment with BC1J and F2 suffered a strong insect attack previous to the setup of the net; therefore, this trial had to be abandoned. In the experiment with BC1N and oilseed rape, the seedling mortality and the absence of flowering in the field were more frequent for the F2 generation (10 out of 38) than for BC1N (10 out of 72) and B. napus (9 out of 53). BC1N plants weighted more than those of B. napus, and F2 had intermediate weight (Table 4). Both BC1N and F2 plants showed lower seed weight, per-seed weight and seed germination percentage than B. napus. The F2 produced less seeds than B. napus, and BC1N had intermediate production (Table 4).

There were significant correlations between seed number and total aboveground biomass (plant weight plus seed weight) for the three plant types ($p < 0.001, \text{Fig. 3}$). The ranking of the slopes of the linear regression equations of seed number in terms of biomass, from the higher to the smaller value, was: B. napus, all phenotypes of BC1N as intermediate, and then F2. Seed number and biomass of F2 showed greater variability than those of BC1N, which were also

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction of F1 hybrids according to the pollination mode (BC1J and BC1N: backcrosses to B. juncea and B. napus, respectively).</td>
</tr>
<tr>
<td>Type of progeny</td>
</tr>
<tr>
<td>BC1J</td>
</tr>
<tr>
<td>BC1N</td>
</tr>
<tr>
<td>F2</td>
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<th>Table 2</th>
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<tr>
<td>Segregation of herbicide-resistant and susceptible plants in BC1N and F2, and Chi-square ($\chi^2$) test against expected Mendelian segregation ratio (NS, not significant, $p &gt; 0.05$).</td>
</tr>
<tr>
<td>Plant type</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td>Data of direct seedling treatment</td>
</tr>
<tr>
<td>F2</td>
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<tr>
<td>BC1N</td>
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<tr>
<td>BC1J</td>
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<tr>
<td>Data inferred from progeny analysis</td>
</tr>
<tr>
<td>F2</td>
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<tr>
<td>BC1N</td>
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</tbody>
</table>

Fig. 2. Histogram of flow cytometry results and estimated Normal distribution curve of BC1N (empty bars) and BC1J (hatched bars).
Table 3
Percentage of resistant plants in the progeny of susceptible B. napus, BC1N and F2 plants in the field experiment.

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Number of plants</th>
<th>Progeny</th>
<th>Percentage of resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>With R progeny</td>
<td>Resistant</td>
</tr>
<tr>
<td>BC1NS</td>
<td>35</td>
<td>13*</td>
<td>48</td>
</tr>
<tr>
<td>F2S</td>
<td>4</td>
<td>4</td>
<td>81</td>
</tr>
<tr>
<td>F2R</td>
<td>47</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Identified as BC1NS* in Figs. 4 and 5.

Table 4
Mean (±95% CI) of plant weight, seed weight, seed number, per-seed weight and germination of F2, BC1N and B. napus.

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Plant weight (g)</th>
<th>Total seed weight (g)</th>
<th>No. of seeds</th>
<th>Per-seed weight (mg)</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1N</td>
<td>33.9 ± 7.8b</td>
<td>2.10 ± 0.78b</td>
<td>1084 ± 450b</td>
<td>1.90 ± 0.11b</td>
<td>60.2 ± 4.5a</td>
</tr>
<tr>
<td>F2</td>
<td>26.1 ± 8.6b</td>
<td>0.90 ± 0.35b</td>
<td>438 ± 155b</td>
<td>1.90 ± 0.17b</td>
<td>67.9 ± 9.5a</td>
</tr>
<tr>
<td>B. napus</td>
<td>17.0 ± 3.7b</td>
<td>3.60 ± 0.87b</td>
<td>1383 ± 342b</td>
<td>2.60 ± 0.10b</td>
<td>79.1 ± 2.5a</td>
</tr>
<tr>
<td>F2S</td>
<td>5.41***</td>
<td>15.81***</td>
<td>10.57***</td>
<td>33.53***</td>
<td>8.86***</td>
</tr>
</tbody>
</table>

F-Values are from one-way ANOVA on individual traits (**0.05 < p < 0.01; 0.01 < ***p < 0.001). Different superscript letters indicate significant differences between mean values within columns for each experiment according to Tukey’s HSD (α = 0.05).

3.4. Correlation to discrete markers

There was no difference of plant weight, seed weight, seed number and per-seed weight between BC1NR and BC1NS plants. However, a different picture appeared when BC1 NS was separated into group BC1NS that did not produce any R progeny and group BC1NS* that produced a few R progeny (see Table 3). BC1NR and BC1NS plants produced higher plant weight, more seeds and seed weight than the other BC1NS plants. The per-seed weight remained unchanged. There was no difference between BC1NS* and BC1NR plants (Fig. 4). The number of seeds in terms of biomass of the BC1NS (excluding BC1NS*) was as low as that of the F2 plants (Fig. 3). Plants with B. napus morphology had higher plant weight, produced higher seed weight and seed number than plants with B. juncea morphology (data not shown). BC1NS* and BC1NR exhibited more plants with B. napus morphology than the other BC1NS (86 and 73% versus 39%, respectively; χ² = 31.2, p < 0.001; Fig. 5). No such a difference was found between F2S and F2R segregants (Fig. 4), but this was perhaps due to the low number of F2S plants tested. The percentage of plants with large flowers increased significantly (χ² = 9.5, p = 0.027) from BC1NS (52%) to BC1NS* (64%) and BC1NR (73%). F2R produced larger flowers than F2S, 27% versus 0%, respectively (χ² = 31.2, p < 0.001, Fig. 4).

4. Discussion

4.1. Hybrids and backcrosses production

Hybrids produced 34% viable pollens on average, a value similar to that found in other studies (0–28% in [13]; 32–35% in [15]), thus allowing self-fertilization to produce F2 generation, and possibly serving for outcrossing. However, most of the viable pollen in the pollen cloud in a mixed stand of B. napus and B. juncea in the fields comes from the two parent species, so that self-pollination should not be the most frequent pollination event. Hybrids F1 receiving pollens from B. napus produced twice as many BC1 seeds as those receiving B. juncea pollens. It is likely that pollens of B. napus possessed stronger fertilizing ability than B. juncea ones [22]. However, the rate of seed set observed here is much lower than that observed in a similar study, which indicates high variability according to genotypes and/or conditions [18].

BC1 produced with B. napus (BC1N) had larger seeds, and germinated better than the F2 and BC1 produced with B. juncea (BC1J). These characteristics, together with a better ability to produce BC1 with B. napus, could be useful in predicting a greater prevalence of these plants, especially at the places where abundant pollens of B. napus occur, i.e. close to oilseed rape fields. The difference of DNA content between the two types of BC1 suggests a rapid differentiation within one generation, by around 2 chromosomes on average, but there was a large variability within each group. Cytological analysis of similar plant material indicated that B-chromosomes in the progeny of hybrids tend to be eliminated more rapidly than C-chromosomes [23], which again suggest a more rapid return to B. napus-like plants.

The expected Mendelian ratios obtained for the resistant phenotype show that this trait is easy to be transferred between the two species and stably inherited in subsequent generations of hybrids whichever genitor is used as female. Such a feature is not uncommon in the transmission of transgenes although they have no DNA counterpart in the recipient species [10,13,24], but distorted segregation was sometimes encountered in different lines [10] or for different transgenes [25]. In our study, the precise location on the A, B or C genome of the ALS gene conferring resistance gene was not known, but non-biased segregation would suggest an A-chromosome. This situation gives rise to the persistence of the herbicide resistance trait in the field because the hybrid gametes always display native or recombined A-chromosomes.
Fig. 4. Plant weight, seed weight, seed number and per-seed weight for BC1 NR and BC1 NS in the field experiment. BC1 NS is separated into group BC1 NS that did not produce any R progeny and group BC1 NS* that produced a few R progeny (see Table 3). p-Values are from one-way ANOVA results. Vertical bars denote SE of the mean. Different letters indicate significant differences between mean values according to Tukey’s HSD (α = 0.05) and Contrast tests.

Fig. 5. Percentage of the two categories of morphology (top) and flower size (bottom) for resistant (R) and susceptible (S) phenotypes of F2 (right) and BC1 N (left) in the field experiment. BC1 NS is separated into group BC1 NS that did not produce any R progeny and group BC1 NS* that produced a few R progeny (see Table 3). B. juncea morphology and small flower size are filled bars; B. napus morphology and large flower size are empty bars. Different letters indicate significant differences according to Fisher’s test (p-values).
which would be not the case if it were on a B or C chromosome.

4.2. Productivity of the resistant progeny

In our study, we evaluated productivity, measured as biomass and seed set parameters, without competition between co-cultivated genotypes, which could correspond to the conditions at the field border (i.e. the cultivated border line that is not sown) and roadside as well. In most studies of transgene escape between *B. napus* and *B. rapa*, *BC1* and *F2* exhibited lower seed production than their parents [6,9]. In our study, *BC1* and *F2* demonstrated significantly higher vegetative performance than *B. napus*, which could provide opportunity for quick establishment at the expense of volunteer and cropped oilseed rape. In contrast, *BC1* and *F2* produced less seeds than *B. napus*, and they had lower seed production efficiency per biomass unit than *B. napus* (Fig. 3) which could impact their reproductive success because more resources were needed to produce seeds. The difference seed production between *BC1* and *F2* was bordering significance, because of the effect of the high variance found in *BC1*, which probably denoted an effective difference. Especially, the productivity of *BC1* per biomass unit was clearly higher than that of *F2* (Fig. 3). Therefore, it is likely that *BC1* are able to establish in waste areas, field borders, and even arable fields, more easily and rapidly than *F2* plants. The low variability noted for *B. rapa* in the regression function between seed number and biomass was of environmental nature only because the cv. Drakkar is a pure line, which suggests that the higher variability recorded in *BC1* and *F2* could be genetically determined. A similar case of higher variability of performances was also observed in *B. napus* and *B. rapa* hybrid progeny [8], which could be the source of further genetic adaptation and lead to successful volunteers or new weeds in both non-crop and crop habitats. Such a situation is highly favorable to the rapid evolution of adaptive traits to the agro-ecosystem [26]. In addition, half of the *B. napus* exhibit the herbicide-resistance, which could help plants to survive in arable fields and roadsidess if the corresponding herbicide were employed.

No difference of plant weight, seed weight, seed number and per-seed weight was observed between resistant and susceptible *BC1* plants (*BC1*NR versus *BC1*NS + *BC1*NS*) and between resistant and susceptible *F2* plants, which suggests that no detrimental effect existed for expressing the herbicide-resistance gene. Preliminary data of the comparison of the mutant line used here with its original cultivar showed that delayed flowering was responsible for 6–14% lower yield [19]. In two other cases only the fitness difference found in *BC1* and *F2* could be genetically determined. A similar case of higher variability of performances was also observed in *B. napus* and *B. rapa* hybrid progeny [8], which could be the source of further genetic adaptation and lead to successful volunteers or new weeds in both non-crop and crop habitats. Such a situation is highly favorable to the rapid evolution of adaptive traits to the agro-ecosystem [26]. In addition, half of the *B. napus* exhibit the herbicide-resistance, which could help plants to survive in arable fields and roadsidess if the corresponding herbicide were employed.

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