Environ. Biosafety Res. 6 (2007) 197–206

DOI: 10.1051/ebr:2007007

Available online at:
www.ebr-journal.org

Transgene escape in sugar beet production fields: data from six years farm scale monitoring

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Concerns have been raised in Europe about the efficiency, sustainability, and environmental impact of the first genetically modified crops. The committees and regulators in charge of approving procedures have encouraged a field trial approach for safety assessment studies under current agronomic conditions. We describe the gene flow from sugar beet (*Beta vulgaris* L.) in a multi-year and multi-crop monitoring study on farmers' fields at two locations that has been carried out since 1995. We analyzed two sugar beet lines that have been genetically transformed for herbicide resistance. One sugar beet has resistance to glufosinate and the other to glyphosate. Large differences among lines, years and locations were observed. These differences provided a broad range of situations to estimate the risks. Sugar beet bolters produced the majority (86%) of the herbicide-resistant seeds harvested in the field. Direct pollen flow from sugar beet bolters to weed beets that were growing within the same field as well as in a neighboring field that was left fallow accounted for only 0.4% of the resistant seeds released over the years and locations. Descendants of the hybrids between the sugar beet and the weed beet produced the remaining 13.6% of resistant seeds. Herbicide-resistant seeds from the progeny of the weed beet were recorded up to 112 m away from the closest transgenic pollen donor. Indications were observed of non-randomness of the weed beet producing resistant progeny. We also analyzed pollen flow to male-sterile bait plants located within and outside of the sugar beet field. Herbicide-resistant pollen flow was recorded up to 277 m, and fitted with an inverse power regression. Using sugar beet varieties with no, or very low, sensitivity to bolting and destroying bolters are two necessary measures that could delay gene flow.

Keywords: transgene / herbicide resistance / pollen flow / farm-scale / sugar beet / *Beta vulgaris*

INTRODUCTION

Genetic engineering is a recent tool proposed by seed breeders to improve crop quality and adapt cultivars to the requirements of growers, industry and consumers. The first transgenic varieties (soybean, maize and oilseed rape) were released in 1995 in the USA and Canada. However, Europe adopted a more cautious approach of commercial releases. In particular, it quickly became obvious that very little was known about the consequences of such field releases at the farm scale. Such consequences could not be extrapolated from America's experience, because European agriculture and landscape are very different. In addition, several questions about the effects of transgenic varieties on the environment were never addressed in field release authorization procedures in America. Therefore, these questions have to be better documented and discussed. The main concern was the value (for use under normal field conditions) of predictions made from the results of previous small-scale experiments carried out in confined conditions. In particular, there was a need to estimate the cumulative effects and long lasting consequences when various transgenic crops are grown for several years on the same field. Consequently, governments encouraged farm scale studies lasting several years (Messéan, 1997; Squire et al., 2000).

In France, the regulatory authorities encouraged the joint action of governmental research institutes, professional associations and industry. This was the so-called GMO Inter-Ictas project, or GMO farm scale study (Messéan, 1997). The program started in 1995. It included an estimation of the technical benefits of using the transgenic varieties compared to classical ones.

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Article published by EDP Sciences and available at http://www.ebr-journal.org or http://dx.doi.org/10.1051/ebr:2007007

Table 1. Crops rotating with transgenic sugar beet in adjacent fields at the two locations. Adjacent fields in the table were adjacent in the location (55 m width separated by 5 m lanes at Châlons, and 50 m width directly adjacent at Dijon).

Location	Year	Field 1	Field 2	Field 3	Field 4
Châlons	1995-96	Oilseed rape	Wheat	Fallow	Sugar beet
	1996-97	Wheat	Oilseed rape	Sugar beet	Wheat
	1997-98	Sugar beet	Fallow	Wheat	Oilseed rape
	1998-99	Fallow	Wheat	Oilseed rape	Sugar beet
	1999-00	Oilseed rape	Sugar beet	Wheat	Wheat
	2000-01	Wheat	Fallow	Oilseed rape	Sugar beet
Dijon	1995-96	Sugar beet	Wheat	Oilseed rape	Fallow
	1996-97	Wheat	Sugar beet	Wheat	Oilseed rape
	1997-98	Oilseed rape	Wheat	Sugar beet	Wheat
	1998-99	Sugar beet	Oilseed rape	Fallow	Wheat
	1999-00	Barley	Wheat	Oilseed rape	Sugar beet
	2000-01	Oilseed rape	Sugar beet	Wheat	Wheat

It also looked at eventual troublesome or harmful effects, if any, on the farming system and the environment. There were three locations in different climatic regions that were studied: Châlons, in Champagne, in the northeastern part of France; Dijon, in Bourgogne, in the eastern part of France; and Toulouse, in Midi-Pyrénées, in the southern part of France. Three different transgenic crops, all displaying herbicide resistance, were assayed: oilseed rape, sugar beet and maize. Conventional winter wheat and fallow fields were included in the rotations (Tab. 1). We report some results here on how the sugar beet performed in the locations of Châlons and Dijon. Herbicide resistant sugar beets were tested in these experiments because they are much desired by the farmer in order to significantly reduce the number of herbicide sprays and labor time.

In the region of sugar beet root production, three kinds of *Beta vulgaris* L. are generally described: sea beets that are wild plants growing along the coastal zones; sugar beets grown by farmers; and weed beets, an annual form occurring in the fields. They are conspecific and completely inter-fertile, and hybrids between sugar and weed beets are annual (Boudry et al., 1993; Ford-Lloyd and Hawkes, 1986; Hornsey and Arnold, 1979). The weed beet has become a serious problem to sugar beet growers because it spreads over the fields, competes with the sugar beet crop for space and nutrients, and disturbs mechanical harvest. Even when contained by specific weed management techniques, it still infests severely 5% of the sugar beet production area in France (ITB, unpublished report), and is present in more than 70% of the sugar beet fields in the United Kingdom (May, 2004). Weed beet cannot be distinguished from sugar beet at early stages of growth, and there is no selective herbicide available that does not kill the crop as well (Johnson and Burtch, 1959; Longden, 1974). Therefore, breeding transgenic herbicide-resistant varieties can also solve this particular problem. Although sugar beet is a biennial crop that is not expected to flower the first year, a few bolting plants do occur per hectare however. These plants are sugar beets vernalized by low temperatures during the early spring, and/or annual hybrids produced by the pollen of surrounding annual wild beets in the seed production areas. Therefore, the escape of pollen to crossbreed with the weed beet is possible. This can result in the spread of herbicide-resistant weed beets. Several studies already confirmed the possibility of such gene flow among beets. It has been reported that pollen flow and flower fertilization of bait plants (Archimowitsch, 1949; Saeglitz et al., 2000) and ruderal beets (Alibert et al., 2005) was possible up to distances of 200 m away from the pollen source. Evidence of effective crosses between sugar beet and weed beet was also reported (Boudry et al., 1993). Effective crosses between sugar beet and sea beet was also observed (*B. vulgaris* and *B. macrocarpa*; Madsen, 1994). However, these data were obtained in specially designed experiments, not in genuine agricultural conditions, and they cannot provide quantitative estimates. Other studies reported on the resulting gene flow inferred by using molecular markers (Andersen et al., 2005; Viard et al., 2004). Our paper focuses on the amount and distance of pollen dispersal within the GMO farm scale studies, and the transgene escape through seed sets of the sugar beet bolters, from 1996 to 2002. We investigated the pollen flow between transgenic lines and weed beets using the herbicide-resistance trait as a

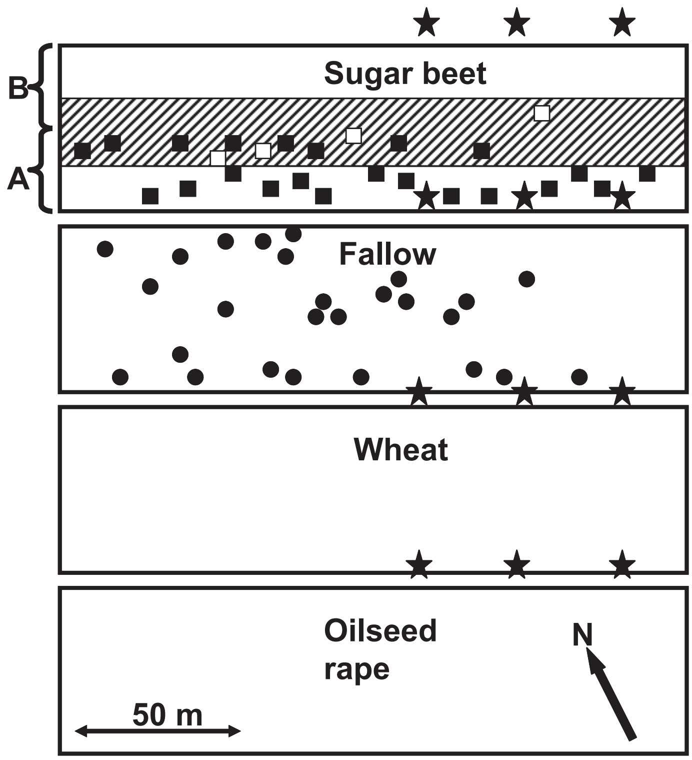

Figure 1. Field design at Châlons in 1998. The sugar beet field was divided into two lanes, each planted with a different transgenic line, A and B. The hatched area was treated with conventional selective herbicides for beet. All flowering plants or groups of plants were mapped: resistant bolters of line A (filled square), susceptible bolters of line A and B (open square), weed beet (dot) and male sterile plants (star).

marker (germplasm resistant to glyphosate or to glufosinate). We also used male-sterile plants located in patches at various distances from the field where the transgenic variety was grown (Fig. 1).

RESULTS AND DISCUSSION

Flowering and seed set

The number of sugar beet bolters ranged from 0 to 121, and was highly variable according to the transgenic line and year. It provided a good opportunity to study the consequences of gene flow, and was used as the marked pollen source in ten of the twelve cases (Tabs. 2 and 3). In one case, roots of one transgenic line growing in the fields that were left fallow were also considered to be pollen donors. Of the two lines, line A was much more prone to flower, which could be because it was an experimental material not designed for commercialization. These flowering plants included sugar beets vernalized by low temperatures as well as annual hybrids. Since the transgenic lines were produced on susceptible male-sterile plants pollinated by a transgenic pollen donor, the vernalized sugar beets were heterozygous and produced both resistant and susceptible pollen. In contrast, annual hybrids originated from the pollination of the male sterile plants by susceptible wild beets, and therefore they were susceptible. Only one herbicide was chosen for testing pollen flow in a given location and year. This was because a seedling-destructive test, herbicide treatment, was used to identify the phenotype, and the number of seeds available was often small. Therefore, there was a contribution to gene flow from one line that was not accounted for in the test. This line was considered to be a donor of susceptible pollen just as the annual hybrids or weed beets were (Tab. 2). At Dijon, the weed beets were always transplanted. In contrast, Châlons had a remote history of sugar beet production, and weed beets emerged spontaneously and grew in the sugar beet and fallow fields. These weed beets in the wheat and oilseed rape fields were destroyed by soil tillage and herbicide programs. They were also controlled by glyphosate or glufosinate sprays in the dedicated areas of the corresponding transgenic sugar beet lines. The onset of flowering

https://doi.org/10.1051/ebr:2007007 Published online by Cambridge University Press

Table 2. Number of sugar beets and weed beets in the two locations. We differentiate among sugar beet bolters, resistant sugar beet bolters of the line whose herbicide resistance was tested in further seedling analysis (glufosinate or glyphosate, arbitrarily indicated by A and B), and susceptible sugar beet bolters (including annual hybrids and bolters of the second transgenic line). The status (resistant/susceptible) is based on the percentage of resistant seedlings in each plant progeny (see text for details). For weed beets, we differentiate weed beet growing in sugar beet field and in fallow.

Location	Year	Number of plants				
		Sugar beet bolters			Weed beets	
		Line	Resistant	Susceptible	In sugar beet	In fallow
Châlons	1996	A	26	33	185[1]	87
	1997	A	117	4	15	0
	1998	A	51	11	0	55
	1999	A	23[2]	0	0	14[3]
	2000	A	0	3	154[4]	0
	2001	A	2 (d[5])	0	3	11
Dijon	1996	A	7	26	28[1]	0
	1997	B	24	12	14[1]	0
	1998	A	46	8	12[1]	0
	1999	A	8	12	53[1]	28[1]
	2000	_	0	0	0	0
	2001	B	13 (d)	0	0	0

[1] Transplanted weed beets. [2] Including 7 volunteer roots growing in the fallow, and 9 plants that were not harvested and left to set seeds in the field. [3] Including 6 plants that were not harvested. [4] Including 41 sowed susceptible and 35 resistant offspring of backcrosses of hybrids between resistant sugar beets and weed beets, and 40 spontaneous weed beet that were not harvested. [5] d: Destroyed.

was roughly synchronous among beet categories, from July 10 to August 5 according to the year and location (data not shown). The flowering period was indeterminate, so overlapping was observed every year.

Male-sterile beets always produced monogerm glomerules (or seed balls) as expected, because they were selected to be the seed parent of certified monogerm hybrid commercial varieties. However, monogermy is a recessive trait (Savitsky, 1954) that is not severely controlled in the pollen donor of certified seeds, so bolters in a root production field release polygerm glomerules. More than 50% of glomerules had two flowers, and the weed beets had up to four. The average number of flowers per glomerule was 1.70 ± 0.17 and 2.06 ± 0.15 for sugar beets and weed beets, respectively. These values were used to weight the number of glomerules produced per plant and to calculate the number of flowers releasing pollen into the field (Tab. 3). The number of flowers per plant ranged from 0 to 19 000 for sugar beet bolters, and 0 to 14 000 for weed beets. The total number of fertile flowers in a location, including sugar beet and fallow fields, ranged from 34 300 to 873 700. Given that resistant sugar beet bolters were heterozygous for the transgene, and assuming that all the flowers produced a comparable amount of pollen, the percentage of pollen carrying the tracked transgene for herbicide resistance was between 2.3 and 49.2% (Tab. 3). However, it is likely that the viability of the pollen from sugar beet bolters was much lower than that of the annual hybrids and the weed beets (G. Alcaraz, personal communication), resulting in a lower actual rate of resistant pollen in the pollen cloud. The percentage of flowers producing a seed varied widely among individuals, from 0 to 98%. It varied also among beet categories and years, from 0 to 30.9% for sugar beet bolters, and from 2.6 to 17.6 for weed beets. On the average, the percentage of flowers producing a seed was 9.8%, which indicated a pollen-limiting condition.

Occurrence of herbicide-resistant seeds in progenies of fertile plants

The number of viable seeds produced by the four categories of fertile beets was estimated by the number of seedlings obtained in greenhouse conditions (Tab. 4). Because of the large variation of the relative location of the donor and target plants in the different years and locations, and the large range of the expected percentage of resistant pollen in the pollen cloud, there was a wide variability of the results in terms of the percentage of resistant seeds produced, even within each category of plant. On the average, 39.6 and 6.2% of the seeds produced by

Table 3. Total number of flowers calculated based on the total number of glomerules multiplied by the average number of flower per glomerule: 1.70 and 2.06 for sugar beet and weed beet, respectively. We also estimated for each year the percentage of pollen carrying resistance assuming all flowers produced the same amount of pollen. This percentage is calculated as half the ratio of the number of flowers produced by resistant (heterozygous) plants over the total number of fertile flowers.

Location	Year	Total number of flowers				% of R pollen
		Sugar beet bolters		Weed beets		
		Resistant	Susceptible	In sugar beet	In fallow	
Châlons	1996	40 200	63 300	641 600	128 700	2.3
	1997	641 500	1970	8820	na[1]	49.2
	1998	81 500	17 500	na	54 300	26.6
	1999	46 300[2]	na	na	10 800[2]	40.5
	2000	na	11 100	464 600[2,3]	na	14.8
	2001	25	na	4810	9260	7.4
Dijon	1996	9580	26 500	87 600	na	3.9
	1997	106 900	57 600	3440	na	31.8
	1998	64 400	23 200	8660	na	33.5
	1999	35 400	88 600	20 000	35 900	9.8

[1] na: Not applicable. [2] Weighted values assuming the non-harvested plants left to set seed in the field had the same flower production as the harvested plants. [3] Including 140 700 flowers produced by the 35 sowed heterozygous resistant plants.

sugar beet bolters and weed beets, respectively, were resistant.

(1) The highest percentage of herbicide-resistant seedlings was observed in the progeny of resistant sugar beet bolters (Tab. 4). This percentage varied from 38.5 to 69.1% according to year and location, with an average of 58.2%. Assuming regular Mendelian segregation of heterozygotes and lack of pollen coming from the other categories of beet, the R:S ratio should be 75:25. The lower frequency of resistance recorded here was probably due to the contribution of numerous susceptible plants to the pollen cloud. There were bolters in the central lane treated with conventional products and in the second transgenic line, and spontaneous or planted weed beets in the sugar beet field and the fields that were fallow. A higher efficiency of pollen from the weed beet could also contribute to this result. This category of plant accounted for 84.8% of the total resistant seed production over the years studied.

(2) Resistant seedlings also appeared in the progeny of 41 out of the 109 susceptible sugar beet bolters. Their percentage was much lower than what was found for the resistant sugar beet bolters, ranging from 0.2 to 12.5, with an average of 1.7% (Tab. 4). The highest value at Châlons in 2000 corresponded to the absence of sugar beet bolters, but the presence within the field of sowed resistant weed beet that could possibly have a high pollination efficiency. However, a high rate of resistant pollen could not warrant a high enough frequency of resistant progeny as was seen at Dijon in 1997 and 1998. Different weather conditions, wind intensity and turbulence, and spatial distribution of the resistant pollen donor plants could account for this variability. This category of plant accounted for 1.2% of the total resistant seed production over the years studied.

(3) In 2000 at Châlons, resistant weed beets were sown and 35 plants flowered. In 2001, three spontaneous resistant plants appeared; they were probably from the seeds left to shed in the same field in 1999. All these resistant weed beet produced, on average, 74% resistant seedlings (Tab. 4). As there was no other source of resistant pollen during these years, this result was exactly the percentage that could have been expected if these heterozygous plants were effectively isolated from surrounding susceptible ones. This category of plant accounted for 6.4% of the total resistant seed production, and 45.7% of the total resistant seeds produced by weed beets over the years studied.

(4) Finally, 107 out of the 613 harvested susceptible weed beets in the sugar beet field and the field that was fallow produced resistant seedlings. The percentage of resistant seeds varied from 0.004 to 16.7% according to the year, location, and field planted in sugar beet or fallow, with an average of 3.5%. The lowest values were observed when the percentage of R pollen in the pollen cloud was lowest (Châlons 1996 and 2001; Dijon 1996 and 1999). The highest value was observed in 2000 at Châlons. This single year accounted for 51.7% of the total seed production by susceptible weed beets over all years and locations. Only 2.6% of the resistant seed released by susceptible weed beets were produced during the other years. Again, this difference between the year 2000 and the others supported a higher efficiency

Table 4. Number of viable seeds resistant to the herbicide, percentage of herbicide resistant seeds (in brackets), and total number of resistant and susceptible seeds calculated for each category of beet each location and year.

Location	Year	Number of viable resistant seeds (and % of R)				Total No. of seed	
		Sugar beet bolters		Weed beets in			
		Resistant	Susceptible	Sugar beet	Fallow	R	S
Châlons	1996	4538 (64.9)	371 (2.7)	3 (0.004)	24 (0.17)	4936	100 337
	1997	27 953 (69.1)	na[1]	14 (5.1)	na	27 967	19 818
	1998	3958 (68.4)	19 (1.1)	na	35 (2.5)	4012	5036
	1999	1641[2] (56.4)	na	na	65[2] (3.9)	1706[2]	2860
	2000	na	125 (12.5)	7442[2,3] (25.4)	na	7567[2]	22 769
	2001	na	na	389 (84.3)[4]	1 (0.4)	390	325
Dijon	1996	749 (38.5)	2 (1.4)	7 (1.4)	na	758	7596
	1997	4339 (68.1)	12 (0.3)	10 (9.4)	na	4361	5477
	1998	889 (56.0)	1 (0.2)	6 (0.4)	na	896	2876
	1999	4828 (44.1)	187 (1.0)	27 (1.3)	15 (0.4)	5057	30 896
Total S		35 038	40 544	101 237	21 171		197 990
Total R		48 895	717	7898	140	57 650	

[1] na: Not applicable. [2] Weighted values assuming the non-harvested plants left to set seed in the field had the same seed production as the harvested ones. [3] Including 3284 seeds (72.9% resistant) produced by the 35 sowed heterozygous resistant plants, and 4158 (16.7% resistant) by susceptible plants. [4] One plant was homozygous resistant and two were heterozygous.

of pollen from resistant weed beets compared to that of sugar beet bolters. All the susceptible weed beets accounted for 7.6% of the total resistant seed production over all years and locations.

On the average, 5765 herbicide-resistant seeds were released every year per hectare of sugar beet. However, half of the total number of resistant seeds was produced in 1997 at Châlons by over-abundant sugar beet bolters. It is likely that farmers would have destroyed these plants because there were obviously too many for good agronomic practices. Globally, sugar beet bolters produced 86% of the resistant seeds, while the weed beets produced only 14%. Pollen flow from resistant sugar beets to susceptible ones accounted for 1.2% of the resistant seeds. In addition, pollen flow from resistant sugar beets to susceptible weed beets within the field and in adjacent fallow fields accounted for 0.4% of the resistant seeds. As a whole, direct pollen flow from the crop was responsible of only 1.6% of the resistant seeds produced in the fields. Subsequent multiplication of resistant weed beets accounted for 13.6% of the resistant seeds.

Characteristics of weed beets producing herbicide-resistant seeds

The largest distance at which a cross was recorded between the transgenic sugar beet bolters and a weed beet was 112 m, at Dijon in 1999, in spite of the low R pollen percentage in the pollen cloud. Since there were no weed beet plants at farther distances, it was not possible to exclude efficient pollen dispersal and crosses from far away. The weed plants that produced resistant offspring were not located at shorter distances from the resistant bolters or farther distances from susceptible plants than the other weed beets. The average distance was at 38 ± 27 m. These plants flowered simultaneously with the other weed plants (not shown). In five instances over twelve cases, they produced more flowers than the plants that did not produced resistant offspring, but that was not a general trend observed (Tab. 5). On average, they set 2.41 ± 0.83 times more viable seeds than other plants, but the Student t test was significant in only three cases. Higher production of flowers and higher seed sets could explain, in part, the capability of those plants to catch more numerous pollen grains and mature more embryos, thus simply having more of a chance to produce resistant offspring. If the number of viable seeds per plant depended somewhat on a genetic factor, the consequence would be the propagation of the transgene together with the most reproductive individuals, a hypothesis that deserves to be taken into account in demographic models. Also, non-random visits of target plants, as already suggested by the presence of various pollinators on beets (Archimowitsch, 1949; Dark, 1971; Free et al., 1975), could be driven by plant characteristics. The role of plant

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Table 5. Comparison of average characteristics of weed beet plants producing resistant offspring *versus* those not producing resistant offspring. We present the number of plant producing resistant seedling (before the slash) or not (after the slash), the number of flower per plant and the number of viable seeds per plant.

Location	Year[1]	Nb of plants R/S	Nb of flowers per plant	Nb of viable seed per plant
Châlons	1996	2 / 183	2554 / 3431	803 / 378
	1996sa	4 / 83	1355 / 1370	162 / 152
	1997	6 / 9	463 / 538	28.9 / 11.4
	1998sa	12 / 43	1244 / 791*[2]	60.8 / 15.6*
	1999sa	1 / 7	385 / 831	57 / 127
	2000	58 / 21	3216 / 1935*	220 / 100
	2001sa	1 / 10	976 / 829	17 / 23.4
Dijon	1996	1 / 27	11 485 / 3127	620 / 180
	1997	6 / 8	181 / 269	13.8 / 4.6*
	1998	2 / 10	1693 / 528*	261 / 110
	1999	11 / 42	519 / 324*	54.9 / 37.2
	1999sa	5 / 23	2637 / 981*	240 / 107*

[1] Year with "sa" means that weed beets were located in the fallow. [2] * Student t test significant at $P = 0.05$, not significant when not indicated.

density, patchy distribution, plant size, distance and respective importance of wind and insect pollination must be further investigated.

Pollen flow to male sterile beets

A large number of transplanted roots of male-sterile plants could not grow or flower together with the other beets, but 395 had simultaneous flowering with the other beets and produced viable seeds. On the average, their progeny were 2.1% herbicide-resistant, which was between the numbers found for susceptible bolters (1.7%) and susceptible weed beets (3.5%). This low percentage was probably due to the greater distance of male-sterile plants to the resistant pollen sources, while resistant bolters and weed plants were most often in the same or adjacent field and therefore pollinated each other more frequently. Susceptible bolters mostly belonged to the second transgenic line or grew in the central lane of the field (see Fig. 1), so they were always farther from the resistant pollen source than the closest male-sterile beets. The abundance of resistant offspring varied in function of the distance to the closest resistant bolter (Fig. 2). The farthest distance at which resistant seeds were detected was 277 m. The number of resistant progeny per plant was the best estimator of the realized pollen dispersal because, as shown above, there was pollen limitation and therefore no competition between resistant and susceptible pollens. The percentage of resistant progeny per plant would have been too dependent on the number of susceptible progeny, which was variable because of the variable location and proximity of weed beet pollen donors. Male-sterile plants were grouped into 17 classes of distance to resistant pollen donors (from 6 to 42 plants each class, on the average 23.2), and the mean values over all the data recorded on the two locations during all the years were used to carry out the regression analysis. The number of resistant offspring followed the equation $y = 65.7\,x^{-0.72}$ (95% confidence limits of the power parameter $= -0.72 \pm 0.60$, corrected $R^2 = 0.48$), with x being the distance to the closest resistant bolter in m. To date, there is no similarly detailed description of the pollen flow of sugar beets in a root production field. Data from Bateman (1947) and Vigouroux et al. (1999) also showed inverse

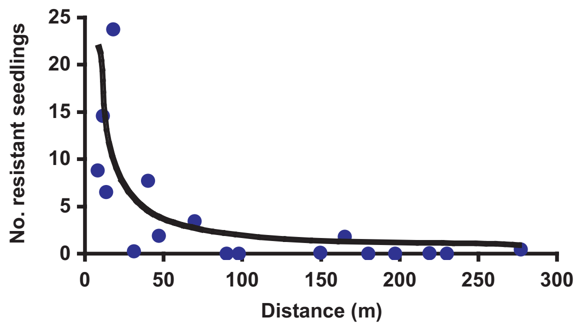

Figure 2. Number of herbicide resistant seedlings produced by male-sterile beets located at various distances from the transgenic field (mean values at 17 distances calculated from 395 plants at the two locations and all years), and regression equation $y = 65.7\,x^{-0.72}$ (corrected $R^2 = 0.48$).

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power regressions, but their data were only recorded over short distances (23 and 15 m, respectively). A specific long-distance experiment confirms this trend (Darmency et al., submitted).

CONCLUSION

The fields were managed in a genuine field environment and provided good practical experience, enabling both farmers and institutes to propose guidelines on how and what to do with the GM sugar beet. They allowed researchers to collect the basic data on the biology of the various categories of beets, including volunteer beets in other crops (not reported here). This will be highly valuable when setting up models to study the influence of the cropping system on gene escape from transgenic crops to volunteers and weed beets (Sester et al., 2007). The conclusions of that study illustrate a wide range of field situations, although it must be clear that most farmers would have reduced the risk of pollen flow by destroying bolters. The rate of sugar beet bolters fell within the range of variation recorded for a sample of commercial varieties in France during the same period, from 0.001 to 0.1% (Perarnaud et al., 2001), but not at Châlons in 1997. Cases with high numbers of sugar beet bolters allowed for the estimation of the consequences of the worst-case scenario. Other practices, such as the usual management of fallow fields could lower the risk even more, as weed beets would be more rare than in the present study.

Confirmed crosses between the sugar beet bolters and weed beet and male-sterile plants were recorded at 112 and 277 m, respectively, indicating long-range dispersal outside the field. The realized gene flow to weed beets was recurrent over the years, although its amount fluctuated according to location and year. The main feature was that most (86%) of the resistant transgenic seeds produced in the fields originated from mother sugar beet bolters. This represented 19.4% of the total seed number released by sugar beet bolters and weed beets together over the years studied. Although obvious, this result strengthens the absolute need to eradicate all transgenic bolters because they produce both the pollen transmitting the transgene to the weed beets and the transgenic seeds that are potentially troublesome. This can be achieved by producing high quality certified seeds that are free of annual hybrid and have nearly no sensitivity to bolting conditions.

If bolters still occur and are not destroyed by farmers, or if transgenic volunteer roots grow and flower in crops subjected to the same herbicide or in fallow fields, the transgenes will unavoidably be transmitted to weed beets with short delay. Such gene flow directly accounted for only 2.6% of the resistant seeds produced by weed beets, and less than 0.1% of the total seed number released by sugar beet bolters and weed beets together over the years. In turn, those herbicide-resistant seeds that resulted from gene flow and were released in the field, which was simulated by sowing backcrossed hybrids of resistant sugar beet and weed beet, were shown to reproduce with a high percentage of resistant progeny. They produced 45.7% of the total resistant seeds released by weed beets, plus 51.7% through pollination of susceptible weed beets, which finally accounted for 97.4% of the total resistant seeds produced by weed beets. If they were not collected for the study, these seeds would be the source of the further multiplication of herbicide resistant weed beets. It would be wise to anticipate such an event and investigate the biology of volunteer beets and weed beets, and model their interaction with cropping systems in order to safely manage the consequences of gene flow. Herbicide resistance is not a unique trait to investigate. Indeed, in cases of transgenes for disease and insect resistance, the widespread presence of such traits in weed beet populations could modify the dynamics of the targeted pests, and serve as a bridge toward sea beets whose ecology could be altered. These questions have been identified as major concerns for several transgenic crops (Stewart et al., 2003).

MATERIALS AND METHODS

Plant materials

The seeds of two transgenic lines were used: a Roundup Ready glyphosate resistant line from Hilleshog, and a Liberty Link glufosinate resistant line from KWS, herein arbitrarily called A and B lines. These lines were experimental material produced by conventional female beets pollinated by transgenic pollen donors. Plants were heterozygous for the transgene. A few seeds could have originated from the pollination of annual wild beets growing in the neighboring section of the nursery, thus resulting in annual hybrids susceptible to herbicide. Male-sterile plants of an experimental germplasm from INRA Dijon were obtained from a greenhouse, and at the stage where the root diameter was 2 cm they were stored at 4 °C for vernalization for at least six weeks before transplanting in the field. Finally, the seeds of weed beets collected in the fields near Dijon were sown in the greenhouse in order to be transplanted in the field at given places. Spontaneous weed beets occurred at Châlons.

Field description

Herbicide-resistant transgenic sugar beets were grown in two of the three locations: near Châlons-en-Champagne, in the north-east of France, and near Dijon, in the east of France, which is the most southern area of sugar beet root

https://doi.org/10.1051/ebr:2007007 Published online by Cambridge University Press

production in France. Each location was about 6−7 ha, including 1 ha field of transgenic sugar beets rotating with transgenic oilseed rape, conventional wheat and fallow fields (Tab. 1). Fields were about 50−55 × 170 m separated by 5 m lanes (Châlons) or adjacent to each other (Dijon) by the largest side oriented N-W/S-E (Châlons) or W-E (Dijon). The sugar beet field was divided along the longest side in two lanes sown with transgenic lines at a density of 10^5 pl.ha^{-1}. In order to check pollen flow, 50 to 100 male-sterile plants were transplanted in different places at the border of the fields (Fig. 1). The experimental release was carried out under authorizations B/FR.95.12.05 and B/FR.99.01.17. There was no commercial sugar beet field within a 500 m radius around the locations.

Herbicide spray was organized along three lanes along the longest side. The central part of the field comprising one half lane of each transgenic line was sprayed the same as conventional sugar beets in the region during the same year, *i.e.* three to six times according to necessity, with various herbicide products including phenmedipham, desmedipham, ethofumesate, metamitrone, lenacil, clopyralid, chloridazone and trisulfuron-methyl. The two remaining half lanes of the transgenic lines were treated with glyphosate and glufosinate, respectively (two times 2 L.ha^{-1} of Roundup Bioforce, 360 g.L^{-1} a.i., and two times 2 L.ha^{-1} unregistered Liberty provided by Bayer Crop Science, 200 g.L^{-1} a.i.).

Plant management

The first year at Châlons and every year at Dijon, weed beets were transplanted at different places throughout the location (Tab. 2). All flowering plants were mapped and the individual onset of flowering (first day of pollen release) was recorded. Plants that germinated or flowered too late to participate in the general pollination period were destroyed. All the seeds produced by beets were carefully collected, plant per plant, so that there was no soil contamination by seeds, except during 1999 and 2000 at Châlons where half of the sugar beet bolters and spontaneous weed beets were left to shed seeds on the soil. In addition, backcrossed seeds of resistant hybrids between resistant sugar beets and susceptible weed beets were sown in the central lane of the field before sugar beet sowing in 2000. The hybrids were those detected, as indicated below, from the seeds collected during the previous years, then backcrossed to susceptible weed beets in order to get 1:1 resistant:susceptible segregating seed progeny. This was carried out in order to simulate the creation of a soil seed bank containing herbicide-resistant seeds, as would have occurred if the seeds were not harvested during the former years.

The fruit of the beet is a glomerule (also called a “seedball”), and is a cluster form with 1 to 6 flowers aggregated during maturation. Each flower forms a cavity called a cell, which contains one seed if pollination is successful. The number of flowers per plant was estimated as the number of glomerules corrected by the average value of flower number per glomerule for each beet category. This value was obtained from 100 counted glomerules of 4−10 collected plants in each category in both locations in 1997.

Analysis of the progenies

All the glomerules, up to 800 for every plant, and up to 4000 when the number of plants was low for a given year and location, were sown in peat in a regulated greenhouse (22 °C day, 18 °C night). Before sowing, glomerules were gently stirred in water for 24 h then dried. The germination rate was used to estimate the total number of viable seeds per individual by extrapolating the number of remaining seeds, if any. The realized pollen flow from the transgenic bolters was estimated by the occurrence of herbicide-resistant offspring in the progeny of susceptible plants. Only one herbicide was chosen for a given location and year, because a destructive test was used, allowing only one check. Young 2−4 leaf seedlings were sprayed with Roundup Bioforce or Liberty at doses of 3 L.ha^{-1} and 5.3 L.ha^{-1}, respectively, using an automatic sprayer delivering spray at 300 L.ha^{-1}. The number of surviving plants was recorded. The frequency of resistant seeds in the progeny of the plants left to shed seed on the soil was assumed to be the same, on the average, as that of tested progeny.

Sugar beet bolters growing in the field in areas where the conventional herbicide program was used could either be resistant or susceptible to glyphosate or glufosinate. We assessed their status based on their progenies’ response to the herbicide. If the 95% confidence limits of the survival rate were lower and not overlapping the 50% rate, the maternal plant was classified as susceptible. Otherwise, the mother plant was classified as resistant. As the pollen most often originated from susceptible plants, this methodology should be effective. However, we could not exclude the possibility that some plants would be misclassified as resistant using this methodology, therefore increasing the estimated percentage of resistant pollen. For the plants that were left to shed seeds on the soil, sugar beet bolters in lanes treated by glyphosate and glufosinate were assumed to be resistant to the herbicide. No plant in the central, conventional lane was left to set seed on the soil at Châlons in 1999. Weed beets were obviously susceptible before any resistant seed stock was raised at Châlons in fallow in 1999 and in sugar beet in 2000. Susceptible and resistant plants were identified on the map and the shortest distances to a susceptible and a resistant counterpart were calculated for every plant. Male-sterile plants at similar distance to resistant pollen donors were grouped and the average values were used for regression analysis of resistant seed set (Systat® 10, SPSS, Chicago).

ACKNOWLEDGEMENTS

The authors wish to thank Marc Fallet and Samuel Garressus (ITB) and Alain Fleury (INRA) for their valuable technical assistance, Hilleshog and KWS for providing the GM material.

Received October 5, 2006; accepted March 5, 2007.

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