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Persistence of oilseed rape (*Brassica napus* L.) outside of cultivated fields

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Abstract It is widely assumed that most cultivated plants cannot persist in natural or semi-natural habitats. Most people thus assume that the plants growing outside of fields (in particular oilseed rape along roadsides) find their origins in the current or previous year's cultivation of that crop. One consequence of this assumption is that the identity of plants growing on road verges is thought to reflect one of the cultivars currently or recently cultivated, while another consequence is the widespread belief that transgenic plants can be simply managed and controlled by stopping their cultivation. Our work shows that this assumption is false. We identify relict plants of a now unmarketable cultivar type of oilseed rape which have persisted in a semi-natural habitat (road verges) for at least 8 years according to farmer surveys in the studied area. More generally, we confirm that the dynamics of the feral oilseed rape plants of road verges is more complex than those resulting from spillage from agricultural machines or from neighbouring arable fields cultivated the previous year. Within the scope of transgenic oilseed rape cultivation, these results suggest that more studies on the dynamics of feral oilseed rape are needed in order to assess more precisely the risks of its invasiveness and its potential impact on genetic pollution between GM fields and non-GM fields.

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Introduction

Gene flow from transgenic crops is today widely studied in order to assess ecological impact and to monitor the deliberate release of GMOs. Pollen flow at the experimental level (Paul et al. 1995; Scheffler et al. 1995; Lavigne et al. 1998) or at the landscape level (Luby and McNicol 1995; Timmons et al. 1995; Squire et al. 1999), and hybridization between crops and wild relatives (Eber et al. 1994; Jorgensen and Andersen 1994; Brown and Brown 1996; Chevre et al. 1997; Darmency et al. 1998), are increasingly documented in the literature on genetically modified crops. However, seed dispersal (Crawley and Brown 1995), potential persistence (Crawley et al. 1993) and invasiveness of crops outside of fields is less documented although often mentioned as a necessary part of risk assessment (Gliddon 1994; Kareiva et al. 1996; Hails 2000). Moreover, these two components would be essential elements in the attempt to establish legal control over transgenic cultivar management at the agricultural scale.

Oilseed rape (*Brassica napus*) is one of the main crops for which transgenic cultivars have been developed. It is also one of the most-invasive cultivated species in temperate countries. While feral populations are common features of road verges, their origin is usually unknown. As for most domesticated plants, the survival of seeds of oilseed rape in the seed-bank is very low in comparison with that of wild relatives (Hails et al. 1997; Chadoeuf et al. 1998). However, a secondary dormancy can be induced in specific conditions (Hails et al. 1997; Pekrun et al. 1998), allowing some seeds to survive for a long time in the soil. Furthermore, population dynamic studies in natural or semi-natural habitats show that the persistence of oilseed rape by local recruitment, without disturbance, is about 3 or 4 years and that the density of feral populations on road verges is correlated with human activities, especially with the transport of seeds by trucks (Crawley and Brown 1995). Within this scope one can ask to what extent these two mechanisms, seed dormancy and persistence above ground, are involved in the settling of feral oilseed rape in natural or semi-natural agricultural habitats. This information will determine: (1) how such populations can influence the spread and persistence of transgenes, (2) which monitoring actions could follow the release of transgenic cultivars, and (3) how these populations should be controlled if an unexpected problem occurred (transgenic feral oilseed rape plants more invasive and/or the source of genetic pollution in fields, for example).

To address this question, we analyzed the chemical content of seeds and the genetic background of feral and cultivated populations of oilseed rape surrounding a grain silo (Selommes, Loir-et-Cher, France) and related the corresponding data to the cultivation history of oilseed rape in the studied area. The cultivars concerned were: (1) before 1973, *erucic* cultivars with a high erucic acid and glucosinolate content in seeds, (2) 1973–1988, *simple zero* cultivars with a low erucic acid and a high glucosinolate content, (3) since 1988, *double zero* cultivars with low erucic acid and glucosinolate content together with *erucic* cultivars for industrial uses. The cultivation history of oilseed rape in the studied area was determined by asking farmers.

Materials and methods

Farmer surveys

In order to obtain information on the agricultural pratices and the local history of oilseed rape cultivation in the studied area, each of the 78 farmers of the 10×10 km^2 area surrounding the silo of Selommes was individualy questioned on the precise location of cultivated oilseed rape and which cultivar they had sown each year between 1993 and 1997. We also noted where they stored their harvest, the route from their oilseed rape fields to the storage location of the harvested seeds (farm, silo of Selommes or other grain silo) and the state of their trailers.

Population sampling

In July 1996, we observed 50 feral sites and 47 fields of oilseed rape along 11 main roads within a radius of 5–8 km from the silo of Selommes. Spatial distribution of feral and cultivated sites were located on a 1:25000 map (see Fig. 1) and seeds were collected within 42 feral sites and 47 fields. Within each field, two or three bulk samples of ten plants in total were collected. Within each feral site seeds were collected on one to ten plants, depending on population size, which ranged from one to more than 100 individuals. Seeds from each feral plant were kept separately.

In 1997, two surveys were performed around the same grain silo. In May, at the flowering time, we observed 65 feral sites along eight main roads which were noted on the 1:25000 map (Fig. 1). In July, we sampled 19 feral sites which were not destroyed before seed maturity. Seeds were collected within each feral site on 1–20 plants depending on population size. In 1997, oilseed rape fields were not sampled due to technical problems.

In both years, each seed sample was divided into two subsamples, one for chemical content analysis (erucic acid and glucosinolates) and the second for greenhouse and electrophoresis analysis.

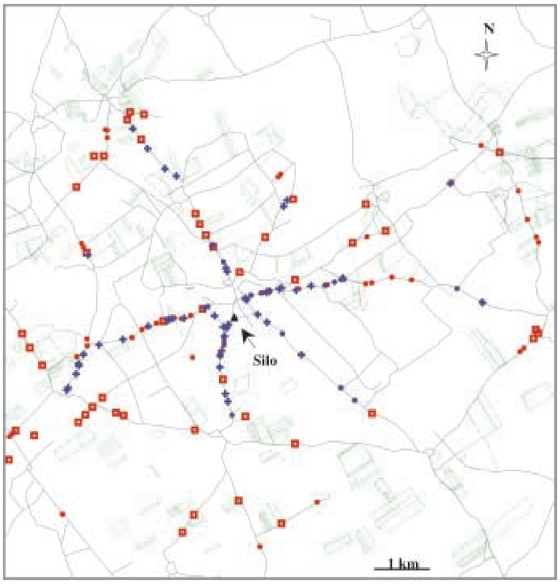

Fig. 1 Spatial distribution of surveyed fields of oilseed rape between 1993 and 1997 (*green lines*), sampled fields in 1996 (*red squares*) and feral sites sampled in 1996 (*red points*) and 1997 (*blue points*) around the silo of Selommes (Loir-et-Cher, France). *Blue crosses* correspond to feral sites surveyed at flowering time

Chemical content of seeds

In 1996, 149 feral plants (42 sites) and 128 cultivated bulks were analyzed to quantify the erucic acid and glucosinolate content of their seeds. In 1997, 115 feral plants were analyzed.

Erucic acid analysis

Seeds were crushed in 5 ml of iso-octane. After shaking and centrifugation, 3 ml of supernatant were dried out with anhydrous Na. Two microliters of fatty acid were esterified with 100 µl of $KOH+CH_3OH$ and 0.5 g of $NaHSO_4.H_2O$ and were shaken for 30 s. After settling, 1 ml of supernatant was analyzed by GPC to quantify the percent of erucic acid.

Glucosinolate analysis

Extraction of glucosinolate was performed two times on 200 mg of crushed seeds in 2 ml of a boiling CH_3OH/H_2O (v/v: 70/30) solution. After centrifugation, supernatants were mixed and adjusted to 5 ml with distilled water. Purification and desulphatation of glucosinolate were performed respectively on DEAE Sephadex A-25 and with 75 µl of sulphatase. After shaking, samples were analyzed by HPLC (Lichrospher CN, 5 µm, 250 mm×4 mm, Merck) and the content of total glucosinolate was detected with PC 1000 software (Thermo-Quest).

Isozyme electrophoresis

Seeds of each sample were sown in the greenhouse. The genotypes of five seedlings per sample were determined by electrophoresis on young leaves for four polymorphic isozyme systems (Leucine aminopeptidase E.C. 3.4.11.1., Aconitate hydratase E.C.4.2.1.3., Glucose-6-phosphate isomerase E.C.5.3.1.9., Malate dehydrogenase E.C.1.1.1.40.). These systems, when combined with the analysis of the chemical content of the seeds, are known to distinguish the cultivar types grown in the studied area until 1997 (*erucic*, *simple zero* and *double zero* cultivar types). Standard horizontal starch-gel electrophoresis was performed following Chevre et al. (1995). We analyzed the progeny of 122 feral plants observed in 37 different sites and of 113 cultivated bulks (41 fields) in 1996, and the progeny of 109 feral plants observed in 19 different sites in 1997.

Cultivar-type determination

Cultivar types of each analyzed sample were determined according to the combined information of the chemical content of their seeds and the isozyme profiles of their progenies. In a first step, samples were subdivided into four classes according to the chemical content of the seeds: the *double zero* phenotypes (percent of erucic acid ≤5% and glucosinolate content ≤40 µmol/g), the *erucic* phenotypes (percent of erucic acid ≥30% and glucosinolate content ≥60 µmol/g), the *simple zero* phenotypes (percent of erucic acid ≤5% and glucosinolate content ≥60 µmol/g) and the *undetermined* phenotypes. These classes are inferred from the chemical-content analysis of current cultivars authorized for human and animal consumption or for industrial uses. In a second step, within each class we compared the expected isozyme profiles and the ones on the five analyzed seedlings per sample. We determined three sub-classes (see Figs. 3, 4): samples in which all progenies had an isozyme genotype consistent with the observed chemical phenotype, samples in which at least one seedling had a hybrid profile between at least one isoenzyme system and the chemical phenotype, and samples in which seedlings mainly had genotypes inconsistent with the chemical phenotype. Among the samples with an *undetermined* phenotype, we expected to observe mainly progenies with hybrids profiles.

Results

Farmer survey

Among the 78 farmers cultivating fields around the silo of Selommes (Loir-et-Cher, France), 39 (50%) precisely located their oilseed rape fields from 1993 to 1997 (Fig. 1) and specified the cultivar types they had sown since 1993 (year of the first PAC declaration in european countries). Surveys show that only two cultivar types of oilseed rape have been cultivated since 1993: *erucic* cultivars for industrial uses (from 7% to 23% of fields depending on the year of cultivation) and *double zero* cultivars for human and animal consumption (from 77% to 93%).

Since 1993, 15 different *double zero* cultivars and only one *erucic* cultivar (Gaspard) have been sown by farmers. In 1996 and 1997, respectively seven and ten different *double zero* cultivars were sown on 90% of the total surface area of oilseed rape cultivation.

The total surface area of oilseed rape cultivated around Selommes has greatly increased from 100 ha in 1993 to more than 300 ha in 1997 (Fig. 2). According to our survey, the oldest record of *simple zero* cultivars dated back to 1988 but only a few farmers were able to give information for dates before 1993.

Cultivar determination

We established an obvious correspondence between the glucosinolate and erucic acid content of the seeds and the isozyme profiles of seedlings both for cultivated and feral oilseed rape samples in 1996 and 1997 (see Figs. 3, 4). Among all the 231 feral samples (1996 and 1997) analyzed, 95% of the observed isozyme genotypes were consistent with the ones expected from the chemical phenotype. Among the 113 bulks sampled in fields, 92% corresponded to cultivar types.

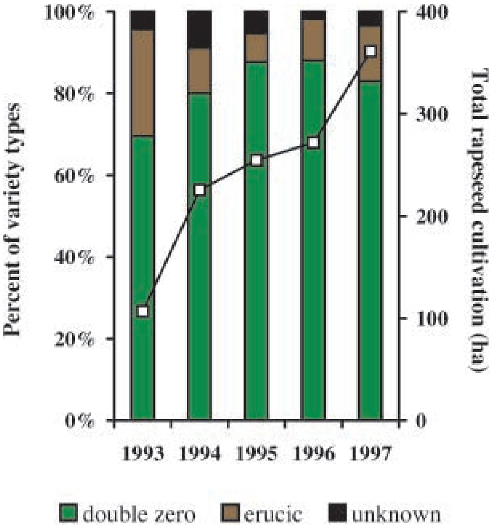

Fig. 2 Proportions of oilseed rape cultivars sown between 1993 and 1997, and change in the total surface area of oilseed rape cultivation according to the farmer's survey

Sampled fields

Among the 41 sampled and analyzed fields in 1996 either repetitions within a field were similar (same chemical phenotype and expected isozyme genotype) or were not similar. Among the 33 homogeneous fields 91% were *double zero* fields and 9% were *erucic*. These results are consistent from surveys with farmers in 1996 (Table 1, χ^2=0.04, 1 *df*, *p*=0.847). The other eight sampled fields showed different combinations of genotypes. We mainly observed mixtures of *double zero* and *undetermined* genotypes within these mixed fields but also two fields containing both *erucic* and *double zero* genotypes (Fig. 3b). Among the 113 bulks sampled in fields in 1996, we observed 93 *double zero* bulks, 11 *erucic* and nine bulks with *undetermined* phenotypes.

Feral sites

Among the 122 feral plants sampled and analyzed (chemical content and isozymes) in 1996 from road verges, 26 were *simple zero* plants (Table 1) although this cultivar has not been sown since 1988 in this area according to the farmers. The *simple zero* plants were collected from 12 different feral sites among which six contained only *simple zero* plants.

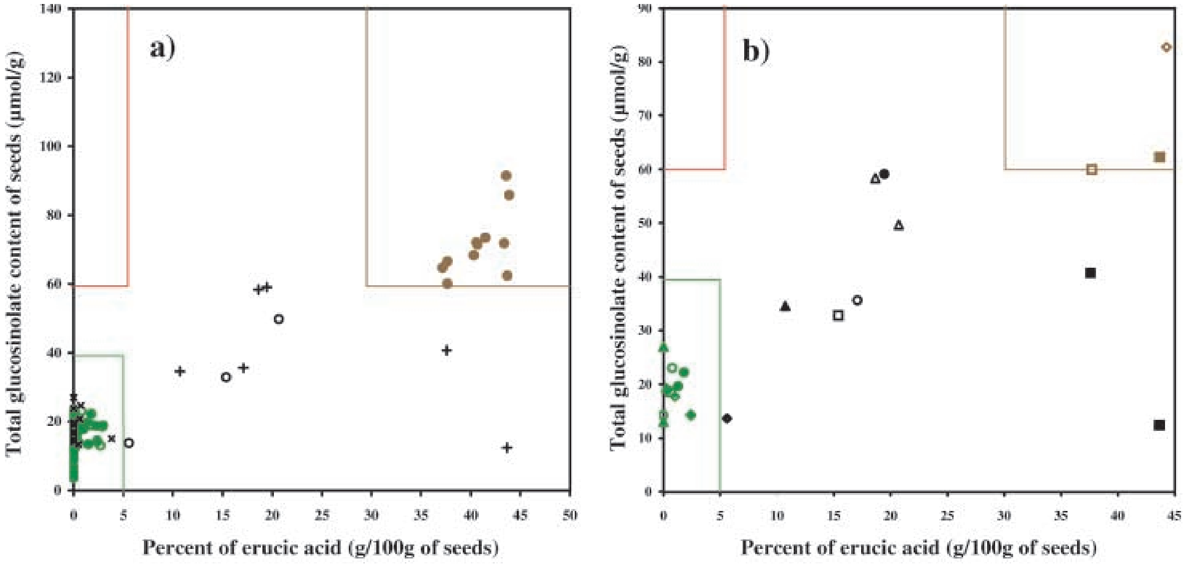

Fig. 3 **a** Scatterplots of combined information on glucosinolate and erucic acid content of seeds and isozyme profiles of seedlings from cultivated samples collected in 1996. ● represent samples for which all five seedlings have the same genotype as the expected one according to phenotype screening. ○ represent samples for which at least one seedling shows a hybrid genotype at one or two loci. + represent samples with inconsistent genotypes according to phenotypes. × represent non-analyzed samples (only chemical contents of seeds). **b** Scatterplots of the glucosinolate and erucic acid content of seeds from bulks sampled from only mixed fields in 1996 (one symbol per field). *Colors* correspond to the three cultivar types: *green* for *double zero*, *brown* for *erucic* and *red* for *simple zero*

Among the 37 feral sites, we mainly observed homogeneous sites with all plants showing the same phenotype and genotype, but 22% of these sites contained a mixture of plants with different genotypes and/or hybrid genotypes (Table 1).

Among the 109 feral plants sampled and analyzed in 1997, three were *simple zero* plants collected from two different sites. Among the 19 feral sites sampled in 1997, we mainly observed homogeneous sites, but 26% of the sites contained a mixture of plants with different genotypes and/or hybrid genotypes (Table 1).

Within the three classes correponding to the three cultivar types (*erucic*, *simple zero* and *double zero*) we observed feral plants with at most one or two seedlings showing hybrid genotypes at one or two loci. The proportion of these plants is always higher among *double zero* plants (42% in 1996 and 15% in 1997) than among *erucic* ones (6% in 1996 and 7% in 1997). Among the *simple zero* feral plants we observed 15% and 0% of these hybrid plants respectively in 1996 and 1997.

In 1996 and 1997, we observed respectively 11% and 4% of feral plants showing an *undetermined* phenotype (Table 1). The seedlings of these plants have mainly hybrid genotypes (Figs. 3-4).

We did not find a direct relationship between the spatial distributions of feral sites of oilseed rape in 1996 and 1997 and the arable fields surveyed since 1993 or sampled in 1996 (Fig. 1).

Discussion

The presence of old cultivars (*simple zero* plants) on road verges in 1996 and 1997 shows that feral plants did not issue solely from fields sown the same or the previ-

Table 1 Number of feral and cultivated sites containing either a single cultivar type or a mixture of more than one cultivar type and/or hybrids (mixed). Number of feral plants observed in the four classes: *erucic*, *simple zero*, *double zero* and *undetermined* phenotypes in 1996 and 1997. *Undetermined* correspond to phenotypes which are not cultivars

Year		Cultivar types			Mixed	*Undetermined*	Total
		Double zero	*Erucic*	*Simple zero*			
1996	Number of fields (survey)	52	6	–	–	–	58
	Number of fields (analysis)	30	3	–	8	–	41
	Number of feral sites	14	5	6	8	4	37
	Number of feral plants	48	35	26	–	13	122
1997	Number of fields (survey)	73	12	–	–	–	85
	Number of feral sites	6	5	1	5	2	19
	Number of feral plants	34	68	3	–	4	109

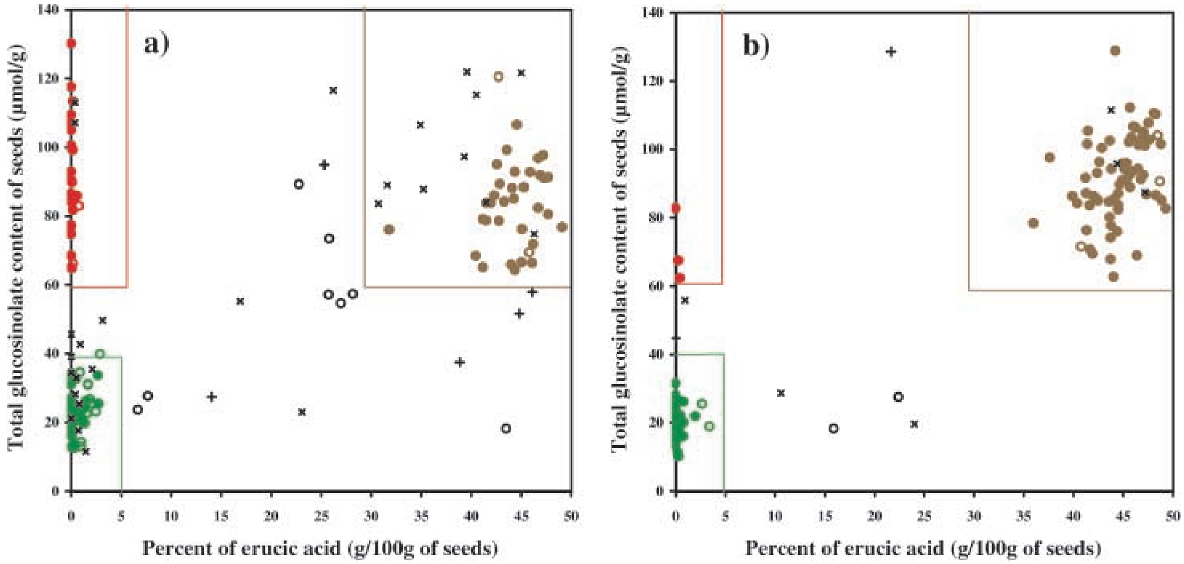

Fig. 4 Scatterplots of combined information on the glucosinolate and *erucic* acid content of seeds and isozyme profiles of seedlings from feral plants in 1996 (**a**) and 1997 (**b**). ● represent samples for which all five seedlings have the same genotype as the expected one according to phenotype screening. ○ represent samples for which at least one seedling shows a hybrid genotype at one or two loci. + represent samples with inconsistent genotypes according to phenotypes. × represent non analyzed samples (only chemical contents of seeds). *Colors* correspond to the three cultivar types: *green* for *double zero*, *brown* for *erucic* and *red* for *simple zero*

ous year, since cultivation of these plants ceased at least 8–9 years ago. These *simple zero* feral plants on road verges could be (1) the result of late germination of dormant seeds from *simple zero* cultivars and/or (2) persisting descendants of populations of *simple zero* plants.

As we observed a low number of *simple zero* plants with at most one or two seedlings exhibiting a hybrid genotype at one or two loci, and since most available pollen has been *double zero* since 1993, persistence above ground of these *simple zero* feral plants appears unlikely. These observations led us to consider that the first mechanism (late germination of dormant seeds) is more probable. It is possible that some farmers grew *simple zero* cultivars after 1988, sowing their own seeds. In any case, farmers would be unlikely have cultivated *simple zero* cultivars after 1990 since seeds containing too much glucosinolate were not marketable by then. Nevertheless, our results are consistent with previous observations which mentioned the persistence of old cultivars of oilseed rape for at least 5–10 years after they were last cultivated (Squire et al. 1999).

In 1996 and 1997, we observed feral sites which contained a mixture of different genotypes (22% in 1996 and 26% in 1997). These observations suggest that the feral populations of oilseed rape could (1) stem from multiple spillages from different fields cultivated the previous year, (2) be delayed germinations of a mixture of genotypes from the seed-bank, or (3) be a mixture of persistent genotypes (local recruitment or late germination from the seed-bank) and seeds steming from trucks or arable fields. We cannot exclude any of these three mechanisms and it is likely that all are true. Moreover, the persistence of feral oilseed rape above ground has been already observed (Crawley and Brown 1995). Because road verges are mowed every year (more than 70% of feral sites observed at the flowering season in 1997 were destroyed before seed maturity), the dynamics and evolution of the feral populations are very difficult to assess at the landscape level. However, their persistence, despite the large proportion of feral populations that are destroyed before seed maturity, suggests a great deal of spread from trucks or from arable fields and/or that a large number of oilseed rape seeds are dormant in the seed-bank.

Literature on the genetic determinism of the erucic acid and glucosinolate content of oilseed rape seeds shows that seedlings issuing from crosses between high *erucic* and low *erucic* oilseed rape have an intermediate proportion of *erucic* acid in seeds (Jourdren et al. 1996). The genetic determinism of glucosinolate content is more complex (Magrath et al. 1993, 1994). However, F_1 seeds issued from crosses between low and high gucosinolate cultivars have a glucosinolate content more or less intermediate to those of the parent lines (Rücker and Röbbelen 1994). Thus, the feral plants with an *undetermined* phenotype (Figs. 3, 4), showing mainly hybrid genotypes, are probably crosses between *erucic* and *double zero* cultivars.

As the samples collected in the fields were bulks of 3–4 plants, the samples with *undetermined* phenotypes could be due to genetic pollution by (1) late germination of volunteer oilseed rape (volunteer *erucic* plants within *double zero* ones, and the inverse) since oilseed rape can persist for several year in the field seed-bank (Lutman 1993) and/or (2) foreign pollen coming from fields cultivated with another cultivar type or from feral plants. As we observed eight mixed fields which contained both *erucic* and *double zero* replicates or one cultivar type and samples with *undetermined* phenotypes (Fig. 3b), we cannot favour one or the other of these two hypothesis without collecting plants separately in the fields. In any case, these observations suggest that genetic pollution between fields over space (pollen flow) or time (volonteer plants) are not insignificant at the agricultural landscape level. This last point is particularly important in order to guarantee GM-free oilseed rape production. Within this scope, our results emphasize the need to determine more precisely whether feral populations are important sources of, and/or corridors for, genetic pollution between fields.

Our results confirm that old cultivars of oilseed rape can persist outside of cultivated fields for at least 8–9 years after they were last cultivated and give evidence that the genetic background of feral plants on road verges does not reflect directly the genetic composition of current cultivated fields. Within the scope of transgenic oilseed rape cultivation, this study suggests that transgenic cultivars could spread and persist in natural or semi-natural habitats even if the transgene does not increase the fitness of the crop. The persistence might be even stronger if the transgene, such as one concerned with insect resistance, increases the fitness of the escaping plant (Stewart et al. 1997). Our ability to predict and understand more precisely the mechanisms (late germination, local recruitment and spread from trucks) which are involved in the spread and persistence of oilseed rape outside of fields will improve our ability to react to unexpected consequences (economic, ecological or those related to health) of large-scale cultivation of transgenic oilseed rape cultivars. Moreover, within the scope of the GM-free production of oilseed rape, it is important to determine if the feral plants on road verges could be sources of, and/or corridors for, genetic pollution between fields. Hence, it appears essential that more studies at the landscape level are required to assess the proportions of these different mechanisms, considering both the dynamics of the feral populations on road verges and their genetic composition.

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