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Long-term persistence of GM oilseed rape in the seedbank

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Coexistence between genetically modified (GM) and non-GM plants is a field of rapid development and considerable controversy. In crops, it is increasingly important to understand and predict the GM volunteer emergence in subsequent non-GM crops. Theoretical models suggest recruitment from the seedbank over extended periods, but empirical evidence matching these predictions has been scarce. Here, we provide evidence of long-term GM seed persistence in conventional agriculture. Ten years after a trial of GM herbicide-tolerant oilseed rape, emergent seedlings were collected and tested for herbicide tolerance. Seedlings that survived the glufosinate herbicide (15 out of 38 volunteers) tested positive for at least one GM insert. The resulting density was equivalent to 0.01 plants m^{-2}, despite complying with volunteer reduction recommendations. These results are important in relation to debating and regulating coexistence of GM and non-GM crops, particularly for planting non-GM crops after GM crops in the same field.

Keywords: volunteer; temporal gene flow; *Brassica napus*; seed; transgene

1. INTRODUCTION

Genetic modification technology makes it possible to engineer organisms with unique trait combinations, and it is currently a challenge to understand the fitness, competitiveness and long-term persistence of such organisms under field conditions (Pilson & Prendeville 2004; Snow *et al.* 2005). In particular, increasing effort is directed towards identifying and predicting the consequences of coexistence between genetically modified (GM) and non-GM organisms (Ellstrand 2003; Pilson & Prendeville 2004). Part of the problem with coexistence is that the inserted transgenes disperse in the environment. The processes with which this happens are analogous to non-GM escapees from agriculture (Ellstrand *et al.* 1999; Ellstrand 2003; Begg *et al.* 2006). The spread of GM organisms into non-GM populations may have implications by affecting the purity of non-GM crops and thus the consumer willingness to buy such products of mixed origin (GM Science Review Panel 2003; Snow *et al.* 2005).

In agriculture, management strategies are adopted to reduce GM volunteer plants (Pekrun *et al.* 1998; GM Science Review Panel 2003). Despite these measures, models now predict problems with volunteers from the seedbank, making it difficult to achieve GM contents below the 0.9% EU threshold (Lutman *et al.* 2005; Begg *et al.* 2006).

In oilseed rape (OSR), *Brassica napus* L., experiments on gene flow between crop varieties are still scarce (Légère 2005). Analysing temporal gene flow through volunteer recruitment from the seedbank is problematic in OSR because it is grown in a crop cycle with a span of only a few years (but see Simard *et al.* 2002; Lutman *et al.* 2005; Messean *et al.* 2007). Long-term GM OSR seed persistence has instead been investigated indirectly by, for example, sowing non-GM seeds in cultivated or non-cultivated soil (Pekrun *et al.* 1998; Lutman *et al.* 2003), seed burial (Schlink 1998), adding seeds to semi-natural habitats (Crawley *et al.* 1993, 2001) or molecular investigations of feral populations (Pessel *et al.* 2001).

In general, studies suggest that the majority of seeds disappear from the seedbank within 2 years (Crawley *et al.* 1993, 2001; Simard *et al.* 2002). Recent models predict over 10-year OSR seed persistence in cultivated soil (Lutman *et al.* 2005; Begg *et al.* 2006), but empirical studies confirming this have not been available (but see Messean *et al.* 2007). Here, we investigated long-term GM seed persistence in a conventionally tilled system. Ten years after a GM OSR trial in Sweden, a field was surveyed and potential GM volunteers detected. Using a combination of crop use history, herbicide application and molecular analysis, we investigated the presence of descendents from the GM field trial.

2. MATERIAL AND METHODS

(a) *Trial with GM OSR in 1995*

In 1995, Plant Genetic Systems N.V. performed a field trial at Lönnstorp Experimental Farm, Sweden (13°06′ E, 55°40′ N) with three transgenic OSR lines (OECD record number SWE95-005). All these three lines were F_1 hybrids between a male sterile line and a fertility restorer line (*barnase* and *barstar* transgenes, respectively; table 1) and carried the transgene *bar*, which confers resistance to the herbicide glufosinate. Four 2×14 m subplots of each hybrid line were sown in a 30×40 m trial plot. The remainder of the plot and a 6–10 m border were sown with conventional OSR. The trial was harvested in autumn 1995 (figure 1), with seed loss prevented as much as possible. Shallow stubble tillage was performed twice to encourage germination before delayed ploughing in late November. Rainfall was sufficient for OSR germination (figure 1).

(b) *Field management 1996–2005*

Between 1996 and 2005, wheat, barley and sugar beet were grown in the trial plot (figure 1). The field was ploughed every year and harrowed before sowing. Volunteer occurrence during 1996–2005 was controlled by herbicides (a mixture of the herbicides tribenuron (Express, DuPont Agro) and fluroxypyr (Starane, Dow AgroSciences)) and subsequent visual inspections. During the first 2 years after harvest, the field was controlled by the Swedish Board of Agriculture, and for two additional years farm staff were under obligation by the Swedish Board of Agriculture to control volunteer rape in the field. During 1996–2005, farm staff controlled any volunteers observed with herbicides before flowering. Subsequent inspections did not detect new volunteers.

(c) *Analysis of OSR volunteers*

Despite volunteer control, volunteers were still observed after 10 years. After harrowing in spring 2005, two persons searched the trial field for 3 hours, collecting all detected volunteers. Volunteers were planted in pots and kept outdoors. As controls we included conventional (Vasaholm: 13°27′ E, 55°38′ N) and feral (Revinge: 13°25′ E, 55°41′ N) OSR plants. Unfortunately, at that time we did not have access to glufosinate-tolerant control plants. After 19 days, plants were hand sprayed with glufosinate herbicide (2% Basta). Spraying was repeated after three weeks. Numbers of surviving plants were recorded after spraying.

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Table 1. The plant genetic systems N.V. (PGS) transgenic lines grown in the trial of glufosinate herbicide (Basta)-resistant oilseed rape in 1995. (Identities of the transgene-carrying plasmids are given within parentheses.)

PGS hybrids	male sterile line *barnase*		restorer line *barstar*
PGS hybrid 1:	Ms1 (pTTM8RE)	×	Rf2 (pTVE74RE)
PGS hybrid 2:	Ms5 (pTCO46RE)	×	Rf2 (pTVE74RE)
PGS1:	Ms1 (pTTM8RE)	×	Rf1 (pTVE74RE)

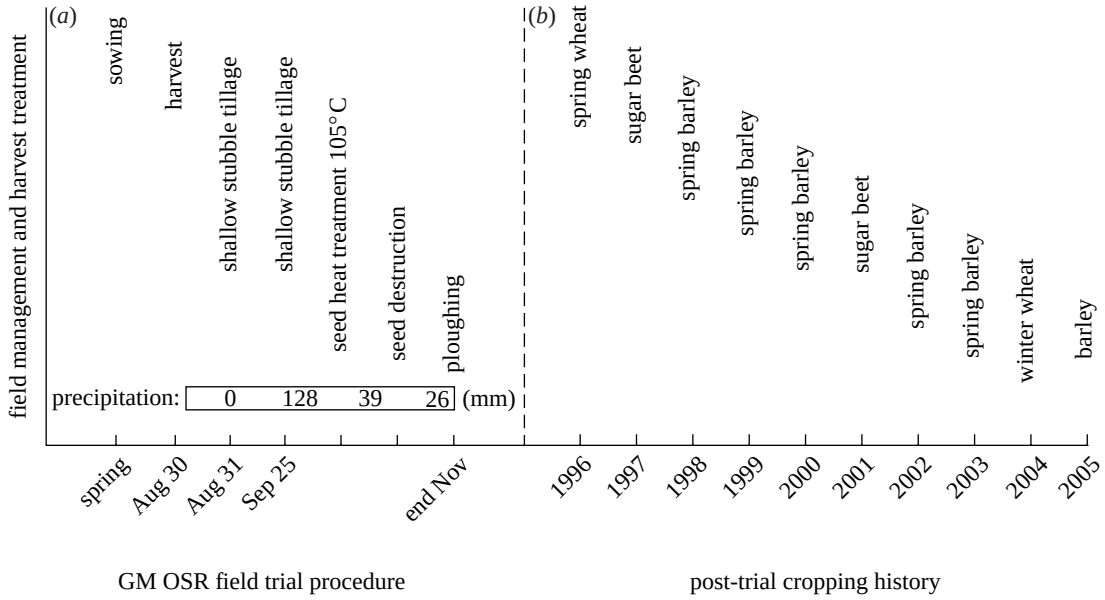

Figure 1. (*a*) Management of the GM Basta-tolerant OSR field trial in 1995, and (*b*) crops grown at the trial site from 1996 to 2005. Numbers in the horizontal bar denote post-harvest monthly precipitation (mm) from August 30 to November 30.

Table 2. Survival of volunteer, crop and feral oilseed rape after glufosinate herbicide (Basta) application, and the frequencies of molecular markers for the transgenes *barnase* and/or *barstar* in the 15 surviving volunteers.

plant type	number of plants	number of plants surviving	plants positive for *barstar*	*barnase*	*barstar*+*barnase*
volunteer	48	15	12	1	2
non-GM crop	67	0	—	—	—
non-GM feral oilseed rape	21	0	—	—	—

Molecular analysis of surviving plants was performed to identify the occurrence of GM lines. One fresh leaf was collected from surviving plants, stored at −20°C and DNA was extracted using the DNeasy standard procedure (Qiagen). PCR analysis was performed with primers (23–24 mers) specific to the inserted constructs, *barstar* and *barnase*, which are genes for male sterility (*barnase*) or its restorer (*barstar*). Positive and negative control plants were included in the PCR analysis. The PCR temperature cycle was 95°C per 4 min (1 cycle), 95°C per 1 min, 57°C per 1 min, 72°C per 2 min (5 cycles), 92°C per 30 s, 57°C per 30 s, 72°C per 2 min (25 cycles) and finally 72°C per 10 min. Products were visualized with ethidium bromide on agarose gels. Control plants with the *barnase* and *barstar* constructs were obtained from other sources and included in the analysis.

In January 2006, 40 soil samples (2.5 cm in diameter and approx. 25 cm deep) were randomly collected in the former trial field. Samples were stored at 2°C until sown in trays and placed in a greenhouse. The soil was watered whenever necessary and mixed several times to encourage germination (Lutman *et al.* 2003).

3. RESULTS

We found 38 volunteer OSR plants in the former trial plot. Fifteen volunteers survived Basta application while none of the controls did (table 2). The difference in survival was highly significant (Fisher's exact probability test, $p<0.0001$). All surviving OSR volunteers were positive for at least one, in two cases both, of the inserted genes (table 2, figure 2), thus clearly demonstrating a link between the GM OSR trial in 1995 and the volunteer population 10 years later. The density of GM OSR was 0.012 plants m^{-2}, and for all volunteers 0.04 plants m^{-2}. Seedlings from seven weed species germinated, but no OSR seedlings germinated.

4. DISCUSSION

Although temporal gene flow has been suggested to make the largest contribution to the mixing of OSR varieties in agricultural fields (Begg *et al.* 2006), data on long-term seed persistence in conventionally tilled fields are rare. Available studies demonstrate that OSR persists for 5–6 or up to 8 years in agricultural fields (Simard *et al.* 2002; Lutman *et al.* 2005; Gruber *et al.* 2007; Messean *et al.* 2007). Our finding

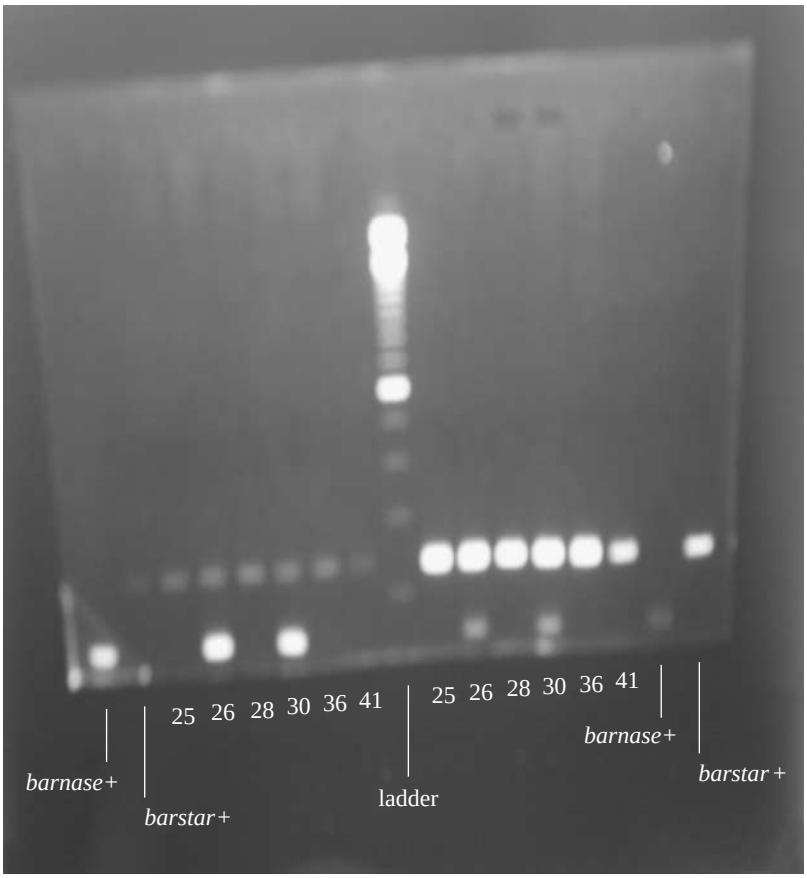

Figure 2. OSR volunteers 25, 26, 28, 30, 36 and 41 tested for *barnase* (left part of the gel) and *barstar* (right part of the gel) genes: *barnase*+ denotes the *barnase* positive control and *barstar*+ the *barstar* positive control. Only volunteers 26 and 30 were positive for *barnase*, but all volunteers (on this gel) were positive for *barstar*.

of transgenic volunteers 10 years after cultivation contributes additional evidence that GM OSR can persist for considerable time in agricultural fields. The data appear to be consistent with theoretical predictions (Lutman *et al.* 2005; Begg *et al.* 2006).

Seed loss at harvest, shallow cultivation and timing of ploughing have been identified as key factors to prevent incorporation of seeds into the seedbank (Begg *et al.* 2006). In the field trial, a protocol based on scientific advice was set up by the Swedish Board of Agriculture to prevent the occurrence of volunteer GM plants, and the trial was meticulously controlled. The shallow stubble tillage performed encourages OSR germination (Pekrun *et al.* 1998), and late ploughing eliminates seedlings (figure 1). Incorporation of seeds into the seedbank was therefore minimized as much as possible.

In the years after the GM field trial, OSR was not grown at the site and volunteers were controlled with herbicides and subsequent observations, so that substantial seed return did not occur. Although every attempt was made to eliminate volunteers, there is a risk that low levels of seed return were possible due to overlooked volunteers. No other trials with GM OSR have been performed at Lönnstorp farm. The extensive control of volunteers makes it possible to conclude that the GM volunteers collected in 2005 most probably were recruited from 10-year old seeds, and provides evidence of long-term GM OSR seed persistence in conventional agriculture. In the year of cultivation, the three hybrid lines probably self-pollinated, hybridized with each other and with the non-GM OSR plants in the trial plot. It is known that restoration of fertility in F_1 lines can be incomplete, and pollen-producing plants had either both the *barnase* and the *barstar* or only the *barstar* (Bisht *et al.* 2004). This could be why the majority of plants had only the *barstar* gene. As only about one-quarter of the trial area (336 m^2/1200 m^2) was sown with GM lines, volunteer density in commercial fields would probably be higher than the 0.01 plants m^{-2} reported here. Also, volunteer control in real fields would never be as strict as in this trial. This finding of volunteers, despite labour intensive control for 10 years, supports previous suggestions (Lutman *et al.* 2005; Begg *et al.* 2006; Messean *et al.* 2007) that volunteer OSR needs to be carefully managed in order for non-GM crops to be planted after GM crops.

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