Schoenenberger and D'Andrea Environmental Sciences Europe 2012, **24**:23
http://www.enveurope.com/content/24/1/23

Environmental Sciences Europe
a SpringerOpen Journal

RESEARCH Open Access

Surveying the occurrence of subspontaneous glyphosate-tolerant genetically engineered *Brassica napus* L. (Brassicaceae) along Swiss railways

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Abstract

Background: Railway tracks represent a highly interlinked habitat with numerous possibilities for accidental entry of oilseed rape due to seed spill during transportation. Moreover, glyphosate is regularly employed to control the vegetation, increasing the possibility of establishment for plants resistant to it. We surveyed the presence of genetically engineered glyphosate tolerant oilseed rape (*Brassica napus*) with a focus on the most important Swiss railway stations. Our objective was to detect accidental establishment of transgenic plants, since Switzerland does not import nor cultivate transgenic oilseed rape.

Results: Seventy-nine railway areas were sampled in Switzerland and the Principality of Liechtenstein, and the feral presence of oilseed rape was detected in 58 of them. A total of 2403 individuals were tested for genetic modification using commercially available immunologic test kits. In four localities, one located in Lugano and three in the area of Basel, a total of 50 plants expressing the CP4 EPSPS protein were detected. In two of the localities, survival of herbicide applications was observed. The populations were probably introduced through contaminated seed spills from freight trains, or during the transfer of goods from cargo ships to trains.

Conclusions: Railways represent an ideal system for herbicide resistant transgenic plants to establish and spread as a result of high selective pressure in favour of herbicide resistance with consequent increased difficulties to keep the infrastructure free of weeds. Crop-to-wild gene flow can occur as several sexually compatible species which are congeneric or in allied genera to oilseed rape were found growing sympatrically. Moreover, the capillary presence of railways in the agricultural landscape provides a putative source of contamination of GE-free agriculture. Our results suggests that carefully adapted monitoring designs may be set in order to detect introduction events that can lead to rapid establishment and growing populations as the accepted contamination thresholds are likely to be biologically insufficient to prevent further environmental contamination.

Keywords: *Brassica napus*, Oilseed rape, Canola, Genetically modified crop, Seed loss and spillage, Environmental contamination, Railway

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Background

General situation

Global cultivation of oilseed rape (*Brassica napus* L.) has been gradually increasing over the last 10 years, reaching about 31.5 Mha in 2010 [1]. In 2011, 26% of the global land area dedicated to oilseed rape was cropped with genetically engineered (GE) cultivars; these cultivars are mainly designed for tolerance to either glyphosate (GLY) or gluphosinate (GLU) [2,3], referred to as genetically engineered herbicide tolerant (GEHT) cultivars. This represents roughly 8 Mha, or 5% of the global biotech crop area. Presently, the import and processing of three GE-oilseed rape cultivars is permitted in the European Union (EU). Of these cultivars, two are authorised as animal feedstuff, whilst the authorisation for use as food is under renewal (events GT 73 and MS8xRF3), whereas one cultivar is currently authorised for processing into both human food and animal feed (event T45). Cultivation is forbidden for any GE-oilseed rape cultivar [4]. Oilseed rape event GT73 is tolerant to the herbicide active substance GLY while the other two to GLU. However, several experimental releases of GE-oilseed rape in the environment were notified in the EU (a total of 379 between 1991 and 2008) mainly in France, UK, Belgium, Germany and Sweden [5].

Situation in Switzerland

In Switzerland, citizens approved a Popular Initiative in 2005, to ban genetically modified organisms (GMO's) from Swiss agriculture for five years [6]. This ban has been extended by three years and is therefore valid until the end of 2013 [7]. Although some GE-Maize and Soybean cultivars are allowed in Switzerland for feed, and some GE-oilseed rape cultivars are tolerated as contaminants (see hereafter) [8], no GE-feed has been imported into the country since 2008 [9]; additionally, no GE-oilseed rape imports are allowed for human consumption [10] Therefore, Switzerland is considered free of any cultivation and import of GE-crops. In 2009, Switzerland produced approximately 70'000 t of oilseed rape seeds; around 11'000 t were imported mainly for oil and biofuel production. Imports originated mainly from Hungary, Romania, Austria, Germany and Balkan countries. Switzerland does not import oilseed rape seeds from countries growing GE-oilseed rape on a large scale such as Canada or USA. Transportation occurs by truck and freight trains [Swiss Federal Office of Agriculture, personal communication]. In Switzerland labelling is required for any product used in the environment (including seeds) containing more than 0.1% authorised GE-cultivars [11], whereas in seed lots up to 0.5% impurities are tolerated for un-authorised varieties that have been approved by another state with a similar procedure as the Swiss [12] or whose environmental compatibility has been verified following [11]. In animal feed, impurities of up to 0.5% unauthorised GMO's are tolerated if these are authorised in the EU, whereas if not authorised in the EU, the Federal Administration can exceptionally allow the use of contaminated (≤ 0.5%) feedstuff upon application, if the contained GE-varieties are permitted in the USA or Canada [13]. Moreover, labelling is required for feed containing more than 0.9% GE-ingredients [13].

Environmental concern

Transgenic crops are being increasingly produced in some parts of the world and imported to most others. A frequently recognized concern is the transfer of transgenic constructs from GE-crops to their wild relatives or to other crops by vertical gene flow, possibly leading to changes in the ecology of crop-related wild species following introgression [14,15]. The introgression into a wild relative of an herbicide resistance gene could lead to increased invasiveness and/or weediness of introgressants [16], with a potentially negative impact on the cropping system [17]. Risks of gene swamping or pollution of natural gene pools, leading to the extinction of wild taxa have also been stressed [14]. Similarly, escape of crop alleles through hybridisation has been shown and is thought to affect a large proportion of domesticated plants, at least in some part of their agricultural distribution [18]. Moreover GE-crops could differ from their non-GE-parent lines not only by the construct inserted, but also through unknown reorganisation of the genome caused by the random process of transformation [19]. Oilseed rape has numerous relatives which are congeneric or in allied genera. Hybridisation can occur with several species [20], most of which can be found as crops, growing subspontaneously in cultivated areas or wild in ruderal habitats.

Cases of environmental escape

Biological characteristics such as partial allogamy and zoogamy, tendency of pod shattering with consequent loss of seeds, ability to survive as volunteers and tendency to form feral populations contribute to making oilseed rape one of the crops with highest likelihood of uncontrolled dispersion of transgenes [21]. For instance, oilseed rape cultivars engineered for GLY or GLU herbicide tolerance escaped soon after their commercial release in Canada [22,23] or recently in the USA [24]. Cases of agricultural contamination by GE-oilseed rape also happened in Europe. In 2000, farmers in France, Germany, Sweden, UK and other European countries inadvertently planted large amounts of contaminated seeds Hyola 401 imported from Canada by the Dutch-owned Advanta Company. In 2007, Deutsche Saatgutveredlung

Table 1 Distribution of the sample sites and detection of transgenic and non-transgenic oilseed rape in Switzerland

Sampling date	Place (railway area)	Coordinates x	Coordinates y	Canton or country	Sample size	Positive samples
16.05.2012	Biel	585157	220177	BE	1	0
16.05.2012	Burgdorf	613671	212235	BE	41	0
16.05.2012	Bützberg	623810	228927	BE	3	0
16.05.2012	Herzogenbuchsee	619887	226449	BE	55	0
16.05.2012	Langenthal	626063	229594	BE	2	0
02.05.2012	Basel, Birsfelden port	615054	267392	BL	95	0
10.05.2012	Muttenz, marshalling yard	616412	264579	BL	29	0
10.05.2012	Muttenz, Auhafen (port area)	616810	265564	BL	47	1
10.05.2012	Basel Bad	612758	268594	BS	4	0
10.05.2012	Basel SBB	611364	266263	BS	7	0
10.05.2012	Basel St. Johann	609915	269330	BS	13	12
02.05.2012	Kleinhüningen, unloading structure (port area)	611308	270495	BS	18	16
02.05.2012	Kleinhüningen, marshalling yard (port area)	611248	269920	BS	58	0
05.09.2011	Nendeln FL	759335	229844	FL	2	0
04.07.2012	Fribourg	577920	183333	FR	2	0
04.07.2012	Romont	559641	171461	FR	9	0
27.06.2012	La Praille	498597	114986	GE	60	0
27.06.2012	Lancy Pont Rouge	498641	116286	GE	18	0
24.10.2011	Landquart	761030	203698	GR	34	0
06.11.2011	Delémont	593355	245675	JU	28	0
27.04.2012	Porrentruy	572996	252459	JU	61	0
15.01.2012	Neuchâtel	562051	205375	NE	7	0
28.04.2012	Serrières	559469	203881	NE	12	0
05.09.2011	Buchs SG	754630	226460	SG	100	0
24.10.2011	Flums	744965	217939	SG	154	0
04.09.2011	Gossau	737042	252783	SG	2	0
04.09.2011	Rorschach	755948	260637	SG	244	0
24.10.2011	Sargans	752606	212333	SG	3	0
05.09.2011	St Margrethen	766129	258112	SG	67	0
04.09.2011	Wil SG	720629	258091	SG	13	0
23.10.2011	Ziegelbrücke	722966	221924	SG	121	0
04.09.2011	Schaffhausen	690376	285153	SH	27	0
04.09.2011	Thayngen	694724	289087	SH	1	0
23.10.2011	Pfäffikon	701556	228891	SZ	14	0
22.01.2012	Bellinzona	722882	117307	TI	9	0
22.01.2012	Biasca	718259	134376	TI	13	0
15.05.2012	Cadenazzo	716087	112364	TI	1	0
15.05.2012	Castione – Arbedo	723658	120471	TI	64	0
06.09.2011	Lugano	716852	95821	TI	23	21*
15.05.2012	Manno	714649	98108	TI	28	0
15.11.2011	Taverne-Torricella	715406	101579	TI	1	0
25.06.2012	Denges	530605	153150	VD	239	0
27.06.2012	Etoy	522313	147602	VD	29	0
15.01.2012	Morges	527479	151538	VD	34	0
04.07.2012	Moudon	551445	168865	VD	35	0

Table 1 Distribution of the sample sites and detection of transgenic and non-transgenic oilseed rape in Switzerland *(Continued)*

04.07.2012	Payerne	562028	185585	VD	5	0
25.06.2012	Renens	534584	154082	VD	200	0
25.06.2012	Yverdon	539084	181527	VD	12	0
23.10.2011	Rotkreuz	675509	221810	ZG	5	0
03.09.2011	Bülach	682490	264443	ZH	42	0
03.09.2011	Dietikon	672700	251498	ZH	31	0
03.09.2011	Dübendorf	689479	250582	ZH	1	0
25.10.2011	Oberwinterthur	699641	262822	ZH	91	0
02.09.2012	Rümlang (field), NC	682521	255467	ZH	5	0
03.09.2011	Seebach	683395	252558	ZH	15	0
03.09.2011	Thalwil	685176	238912	ZH	21	0
03.09.2011	Wezikon	702345	241635	ZH	20	0
25.10.2011	Winterthur	696838	261918	ZH	50	0
23.10.2011	Zürich Herdern-Hardbrücke	681789	248627	ZH	77	0
Total					2403	50

Coordinates are expressed in the Swiss metric coordinates system; contry: FL = Fürstentum Liechtenstein; cantons: BE = Bern, BL = Basel Landschaft, BS = Basel Stadt, FR = Fribourg, GE = Genève, GR = Graubünden, JU = Jura, NE = Neuchâtel, SG = St. Gallen, SH = Schaffhausen, SZ = Schwytz, TI = Ticino, VD = Vaud, ZG = Zug, ZH = Zürich; NC = Negative Control.
* A specimen was deposited at the Herbarium of the Museum of Natural History in Lugano, Switzerland (LUG 19325).

sold contaminated seeds planted over at least 1500 ha [25, with more examples]. Moreover, GE-oilseed rape populations thrive along transport axes even in countries, such as Japan, where no GE-oilseed rape is grown [26]. Despite growing evidence and concern for the escape of GE-cultivars into the wild, monitoring surveys assessing transgene presence in feral populations in the European Union are lacking [27].

The aim of this study was to establish whether GE-GLY-tolerant oilseed rape have established on Swiss railway tracks. Railway tracks represent a highly interlinked habitat with numerous possibilities for seed spill during transportation. Moreover, GLY is regularly employed (up to twice a year) to control the vegetation, hence increasing habitat suitability for plants resistant to this herbicide.

Methods

Railway stations and yards throughout Switzerland were visited during 2011 and 2012 (Figure 1, Table 1) and feral oilseed rape was sampled comprehensively. In case of small populations (generally < 100 individuals) all detected plants were sampled. In the few very large populations detected, as e.g. in Rorschach (Table 1), samples were collected at regular distances of a few meters over the whole area were oilseed rape was growing. Freshly collected leaves were pooled (up to 6 individuals) and ground in a mortar and pestle in demineralised water or extraction buffer provided by the manufacturer of the test strips used (see hereafter). Supernatant liquid was transferred into plastic tubes and analysed with the Reveal® for CP4 (Roundup Ready®) lateral flow strip test kit by Neogen Ltd. Glasgow, UK, or the Envirologix® QuickStix Kit for Roundup Ready® oilseed rape Leaf and Seed, distributed also by Neogen Ltd. The immunologic tests detect CP4 EPSPS, conferring glyphosate tolerance. Both kits were used according to the manufacturer's instructions and gave the same results. According to the same instructions, limit of detection in bulked samples of both the test strips used is 0.1%. Before starting field work, the efficiency of the immunologic tests was confirmed in a laboratory using oilseed rape plants from a commercially grown field near Rümlang, Zurich, as negative control (Table 1), whereas the positive control consisted in certified reference 0304B Event GT73/RT73 oilseed rape Seed (Monsanto) by AOCS, Champaign, IL, USA. Positive reference seed was germinated in a petri dish and then transplanted in a soil-filled pot. Clearly readable positive test strip results (detection of CP4 EPSPS) were possible in a bulk of up to 12 leaves of which one was transgenic. During field work, the bulk samples collected in the railway areas which were positively tested, were tested again individually in order to retrieve the frequency of transgenic plants in single populations. A part of these positively tested samples were sent to the Cantonal Laboratory in Basel, Switzerland, for genetic analyses, particularly real time PCRs of the *gox* and *cp4epsps* transgenes and an event specific PCR of GT73 glyphosate tolerant oilseed rape, confirming the positive results generated with the test strips (data not shown). Herbarium vouchers were deposited at the Herbarium of the

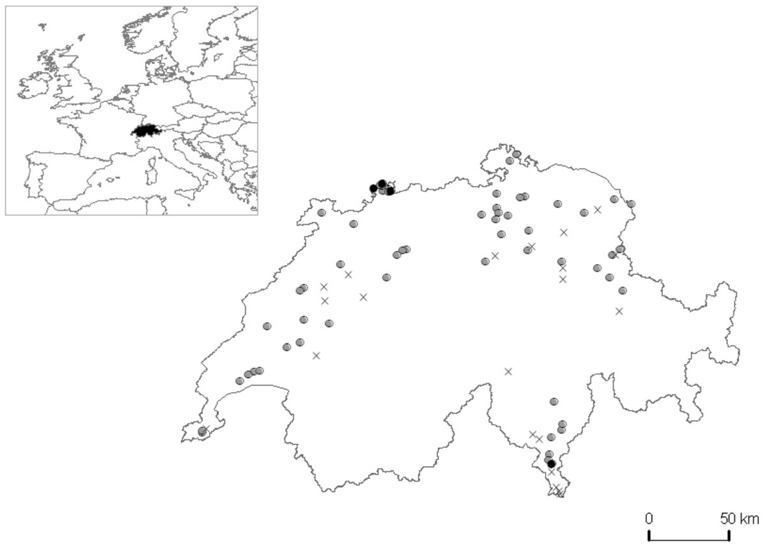

Figure 1 Investigated Railway stations and yards in Switzerland. Grey dots: negatively tested sites; Black dots: positively tested sites (Lugano and Basel); X: railway areas where no oilseed rape was detected.

Cantonal Museum of Natural History in Lugano, Switzerland (Herbarium code: LUG).

Results and discussion

A total of 79 railway stations and areas in Switzerland and in the Principality of Liechtenstein were visited in 2011 and 2012 (Figure 1). The feral presence of oilseed rape was detected in 58 sites (73%) and a total of 2403 individuals were tested for genetic modification (including negative control, Table 1). The presence of weedy oilseed rape in most of the locations is not surprising as oilseed rape has been transported for many years and populations are well established in many Swiss railway areas. In 21 stations (Airolo, Balerna, Bern, Bulle, Chiasso, Chur, Eaux-Vives, Glarus, Ins, Locarno, Lyss, Magadino - Vira, Melide, Mendrisio, Murten, Näfels, Rapperswil, Schaan, St. Gallen, Wattwil, Zug) we were unable to find any oilseed rape plants (Figure 1). The CP4 EPSPS enzyme was immunologically detected in 50 wild growing oilseed rape plants (2.1% of total) distributed over 4 populations (Table 1). At the railway station of Lugano (Canton Ticino) 91.3% of the plants were tested positive, at the unloading railway yard in the port of Kleinhüningen (Canton Basel Stadt) 88.9%, at the railway station of St. Johann (Canton Basel Stadt) 92.3% and at the railway yard in the port Muttenz-Auhafen (Canton Basel Landschaft) 2.1%. All other tested sites were free from GE-oilseed rape. The detection of CP4 EPSPS and the fact that the GE- plants survived GLY treatment in Lugano (which took place in early September 2011) and in Kleinhüningen (in Mai 2012) suggest that the plants were expressing the transgenes at efficient levels. Although herbicides are regularly applied to all railway infrastructures in Switzerland to keep them functional, we did not directly observe treatments in St. Jakob and Muttenz. Only a subset of Swiss railway stations was sampled; nevertheless GEHT plants were found in four locations. This indicates that introduction events of GE-oilseed rape are still a rather rare event in the country and seem to happen predominantly in its important entry points of imported goods, like the Basel area. Transgenic oilseed rape is only recently become available and is transported in much smaller quantities than the conventional crop, and consequently has a lower incidence in railway ruderal habitats. However, it is conceivable that it is just a matter of time for transgenic populations to become more abundant considering the positive selective pressure, with consequent increased difficulties to keep the infrastructure free of weeds. A systematic long-term monitoring is therefore strongly recommended. The GE-populations did not establish through introductions of pure GMHT seeds as illustrated by the population in Muttenz, where only a low percentage of the plants was transgenic. Moreover, had all founder seeds been transgenic in populations with a high frequency of GE-plants (i.e. Lugano, Kleinhüningen and St. Johann), the presence of individuals not containing *cp4 epsps* genes would hardly be possible, as commercialised GE-oilseed rape contains the transgenes in a homozygous state, ruling out the possibility of genetic drift. More likely, the GE- populations established through introduction of few or even a single seed containing transgenes, followed by multiple cycles of reproduction.

The potential for persistence of GLY-tolerant genotypes at the expense of non-transgenic genotypes has been demonstrated experimentally, even in a scenario of GLY drift from field margins [28], and not direct application as it is the case on railway tracks. In Lugano, Kleinhüningen and St. Johann, the GMHT populations most probably underwent strong selection for the transgene, resulting in an enrichment of *cp4 epsps* genes in the population. Moreover, when positively selected by environmental conditions, not only the establishment of transgenes in a given population will increase, but its spread to other feral populations and possibly wild relatives could assume greater importance, too. In Ticino, oilseed rape has not been cultivated for several years [Office for agriculture of the Canton Ticino, personal communication]. However, we detected feral populations in several locations during a floristic survey of the Canton's railways in 2001–2003 [29,30]. At that time, no oilseed rape was growing at the railway station in Lugano, indicating that the detected GMHT population originated after 2003.

Our observations indicate that GE- oilseed rape may be capable of establishing self perpetuating populations outside agricultural areas, for instance on herbicide-treated railway tracks, infrastructures for the transfer of goods from ship to train, and surrounding disturbed habitats in Switzerland. Furthermore, these populations that are persistent from year to year may be capable of hybridizing to produce novel genotypes. GE-oilseed rape has been previously found growing along transport axes [24,26]. However, besides a single GE-oilseed rape plant found in the environment in Belgium [31], our results provide the first evidence of such an event in Europe, and put into perspective the assertion that "seed import spills of oilseed rape imports possibly containing GE-material will be mostly confined to port areas" [27]. Although oilseed rape seeds can be transported by birds and mammals or even dispersed through fireworks, feral oilseed rape growing on railway tracks most probably originate from seed spills during transportation or the transfer of goods. The persistence of a population in a given location is attributed to replenishment through fresh seed spill, emergence from the soil seed bank, shedding from feral mature plants, or to dispersal of feral seeds from one location to another [27]. As regards to the seed bank, studies in the northern hemisphere have reported viable seeds of oilseed rape persisting in disturbed soils for at least 5 years and possibly up to 10 years or more in undisturbed soil [32-34]. As a consequence, we suggest that every location where GE-oilseed rape is found in Switzerland, monitoring and eradication should be carried out during a period of at least 10 years, as the elimination of feral unauthorised GE-populations is a legal requirement [11].

The opportunity to be transported from one available habitat to another is a key factor that could enable further colonization. Indeed, roads and railways interconnect anthropogenic ecosystems and facilitate the spread of plant species adapted to these [35]. This study provides further evidence to the general concern that transport axes act as a possible escape route for GE-plants [36].

Gene flow

The possibility of vertical gene flow between oilseed rape plants, with wild relatives and with domesticated species has been extensively discussed in literature [e.g. 20,21,37]. Significant outcrossing between oilseed rape has been reported up to 100 m, and exceptional outcrossing up to 26 km (reviewed in [21]). In Switzerland, railways often run in agricultural areas; such gene flow from GE-oilseed rape present on the railway infrastructure to adjacent organic and conventional agricultural fields may be of concern, particularly if GE-populations were to increase substantially under strong selection pressure [38]. Oilseed rape is not grown for seed production in Switzerland [SwissOlio Bern, personal communication], and therefore an eventual contamination of agricultural fields would mostly be interrupted by harvesting and processing. Nevertheless, the negative publicity connected with findings of uncontrolled GMO's in the environment could seriously damage the Swiss oilseed rape industry, considering the high sensitivity of consumers regarding GE-food at the European scale [39].

A further source of concern is the potential for hybridisation with related species. In fact, many of the sexually compatible species cited in [27] can be found in ruderal habitats in Switzerland [40]. In particular, several of them were detected on the railroads of Ticino (e.g. *Brassica oleracea* L., *Brassica rapa* L., *Diplotaxis muralis* (L.) DC., *Diplotaxis tenuifolia* (L.) DC., *Erucastrum gallicum* (Willd.) O.E. Schulz, *Raphanus raphanistrum* L., *Rapistrum rugosum* (L.) All.), and often grow sympatrically with oilseed rape [29,30]. For most of these species, hybridisation frequencies with oilseed rape are very low, but introgression can occur significantly with *B. rapa* (reviewed in [41]). However, under strong selection pressure such as regular herbicide application on railway infrastructure, a rare hybridisation event may have significant biological consequences. For instance, a previous study on the prickly (wild) lettuce (*Lactuca serriola* L.), an almost completely autogamous species with low hybridisation rates with its cultivated counterpart *Lactuca sativa* L. (tens of times lower than between oilseed rape and *B. rapa*), showed that even rare hybridization events could have important consequences on the introgression of conventional and/or GE-traits even in absence of continued hybridization [42]. Railway habitat may act as a catalyst of gene flow from GMHT oilseed rape to its wild relatives. Moreover, *B. rapa* may act as a bridge for gene flow towards species with low hybridization frequencies with oilseed rape, but that are sexually compatible with *B. rapa*.

Implication for the setting of contamination thresholds

In Switzerland, the confinement to avoid uncontrolled spread and reproduction, and to rule out the possibility of gene flow to the wild flora, is a legal imperative, independently of the approval status of a GMO [11]. Moreover, the finding of environmental contaminations will raise the question of bearing the financial responsibility of decontamination, potential harm to the environment and the environmental follow-up of the contaminated sites, presently borne by the state. Current contamination thresholds [12,13] represent a political trade-off and are unable to avoid naturalisation of weedy GE-populations established through seed loss during transportation. Our findings and considerations imply that these politically-crafted thresholds are not of sufficient biological relevance.

Conclusions

With this study, we provide early evidence of uncontrolled spread of GE-plants in the environment in Europe. In particular, we detected four feral populations of transgenic oilseed rape on Swiss railways. Although Switzerland neither grows nor imports GE-oilseed rape, it was possible to detect its feral presence at hand of a small scale project, suggesting that contamination can occur also outside the port environment as previously suggested. The ubiquity of railways in the European landscape, corroborated by positive selective pressure and widespread presence of closely related species, raise concerns about genetic contamination of natural environments. We strongly recommend that carefully adapted and systematic long term monitoring designs may be set in order to detect introduction events that can lead to rapid establishment and growing populations. We conclude that contamination thresholds are likely to be biologically insufficient to prevent environmental contamination and bear the potential to legally allow genetic pollution of the environment defeating the real purpose of the legislation, with consequences that are difficult to foresee. More specifically to Switzerland, a leading country in the acquisition of organic farming, where consumers are highly sensitive to the issue of GE-farming, the potential damage of uncontrolled spread of GEHT plants may be severe.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NS and LDA authors contributed equally to the present work. Both authors read and approved the final manuscript.

Acknowledgements

We are grateful to Mirco Hecht and Claudia Bagutti from the Cantonal Laboratory in Basel (Switzerland) for carrying out molecular analyses, to Michele Abderhalden from the Cantonal Museum of Natural History in Lugano (Switzerland) for helping with the manuscript and allowing us to use the facility, to Claudio de Sassi and to four anonymous reviewers for their valuable comments, and to the Swiss Federal Railways for access to their infrastructure. The project was partially funded by AWEL, Section of Biological security of the Canton Zurich, the Fondation pour une Terre Humaine, and by Greenpeace Switzerland.

Received: 12 March 2012 Accepted: 26 August 2012
Published: 11 September 2012

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doi:10.1186/2190-4715-24-23
Cite this article as: Schoenenberger and D'Andrea: **Surveying the occurrence of subspontaneous glyphosate-tolerant genetically engineered *Brassica napus* L. (Brassicaceae) along Swiss railways.** *Environmental Sciences Europe* 2012 **24**:23.

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