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Development of a Monitoring Plan for the Accidental Dispersal of Genetically Modified Oilseed Rape in Italy

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Abstract: This paper presents a pilot project conducted by ISPRA and ARPA Campania to develop a monitoring protocol to detect the presence of genetically modified (GM) oilseed rape (*Brassica napus*) plants resulting from accidental seed dispersal during transportation from entry points to storage and processing facilities; the project has been implemented in Italy's Campania region. The unintentional dispersal of GM oilseed rape seeds and the potential establishment of feral populations have been identified as environmental concerns in various countries, even when GM oilseed rape is imported solely for processing and not for cultivation. The project activities were designed, taking into account the characteristics of the Italian environment and infrastructures. Multiple sampling campaigns were conducted in autumn 2018, spring 2019, and autumn 2019 to validate the selected transects and assess the presence of *Brassicaceae* species, with a particular focus on oilseed rape. These efforts involved direct monitoring and sample collection along transport routes from the port of Salerno to seed companies in the provinces of Benevento and Caserta. Field observations and import data revealed a decrease in oilseed rape movement at the port of Salerno in the years preceding the survey, while seed companies near Benevento remained active sites for white mustard (*Sinapis alba*). The presence of *S. alba* and the simultaneous occurrence of oilseed rape and Raphanus raphanistrum—a species with high hybridization potential—support the hypothesis that seed companies may act as hotspots for accidental seed dispersal and that potential interspecific gene flow can occur. The study also validated the adopted sampling and molecular analysis methods, including DNA extraction and PCR, for the detection of the Cruciferin A (CruA) gene in all Brassica species collected. These findings highlight the need to strengthen post-marketing monitoring plans, even when GM rapeseed is imported solely for processing, to mitigate the potential risks associated with unintended gene flow.

Keywords: GMO; environmental risk assessment (ERA); monitoring; seed dispersal; oil seed rape; *Brassica napus*; *Brassicaceae*; wild relatives; interfertile species; Italy; post-market environmental monitoring (PMEM)

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1. Introduction

In the European Union, the deliberate release of Genetically Modified Organisms (GMOs) into the environment is regulated by Directive 2001/18/EC [1] (the Directive),

while the placing on the market of GMOs for food and feed purposes falls under Regulation No 1829/2003 [2] (the Regulation). A case-by-case Environmental Risk Assessment (ERA) and a Post-Market Environmental Monitoring (PMEM) are key elements to obtain the authorization for the release of GMOs. The ERA identifies and evaluates potential risks—whether direct or indirect, immediate or delayed—to human health and the environment arising from the environmental release of a specific GMO. It considers the GMO's characteristics (such as the recipient organism and the genetic modification), the intended use, and the conditions under which environmental exposure to the GMO may occur.

The PMEM, edited according to Annex VII of the Directive, may include: case-specific monitoring to confirm the occurrence of potential adverse effects of the GMO or its use identified by environmental risk assessment, focusing on their impacts, and general surveillance intended to detect unforeseen adverse effects of the GMO or its use on human health or the environment, including indirect, cumulative, delayed, and long-term effects that were not anticipated in the ERA. Annex VII was supplemented by the Council Decision 2002/811/EC [3] establishing notes providing detailed guidance on the objectives, general principles and design of the PMEM.

The European Food Safety Authority has developed specific guidelines to support the notifier in developing both ERA and PMEM of GMO products [3,4].

The PMEM is developed and implemented by the notifier, who must submit an annual report on the results of the monitoring activities to the European Commission (EC). European Union (EU) member states may adopt additional monitoring and inspection measures, in agreement with the notifier, to supplement the notifier's monitoring plan.

Currently, a limited number of GM plants, including cotton, maize, soybean, oilseed rape, and sugar beet, are authorized for food and feed purposes, but not for cultivation in Europe, while only one plant is authorized for cultivation (maize MON810 renewal process ongoing) (see the GMO register (https://food.ec.europa.eu/plants/genetically-modified-organisms/gmo-register_en (accessed on 16 April 2025)).

Among the authorized products, particular attention shall be given to oilseed rape (*Brassica napus*) due to the high levels of imports into Europe from countries where GM oilseed rape is cultivated and the biology of the species. In the European Union, there are 14 oilseed rape events authorized for placing on the market (import and processing in food and feed, but not for cultivation). Even if cultivation is not foreseen, the potential environmental risks resulting from the accidental release of viable seeds into the environment shall be assessed when performing the ERA.

Brassica napus L., a member of the *Brassicaceae* family, is known for its cruciform flowers and dark bluish-green foliage. This species includes two main types: oil-yielding oleiferous rape (called "canola") and tuber-bearing swede (rutabaga). The oleiferous variety can be further categorized into spring and winter forms. Historical references date back to ancient Sanskrit texts (2000–1500 BC) and later Greek, Roman, and Chinese writings (500–200 BC) [5]. Domestication in Europe likely began in the early Middle Ages, with commercial oilseed rape plantings recorded in the Netherlands by the 16th century, initially used for lamp oil and later as a lubricant for steam engines.

Modern varieties of *B. napus* are characterized by low erucic acid and glucosinolate content, making them suitable for high-quality vegetable oil and animal feed. The winter annual form requires vernalization for successful flowering and seed production, while the spring biotype, found in North America and northern Europe, does not require vernalization but generally yields less than its winter counterpart.

Nowadays, oilseed rape is primarily cultivated in Canada, the United States, the United Kingdom, Germany, France, and the Netherlands as forage for livestock, a source of

edible vegetable oil, and as a fuel for biodiesel. In Italy, it is mainly found in the northern regions as a summer–autumn sown forage crop and for grain production.

Oilseed rape is considered a pioneering species with invasive and weedy tendencies, capable of producing numerous seeds that can disperse over long distances and remain viable for up to 10 years [6,7]. Additionally, oilseed rape can grow in highly degraded areas such as roadsides, railways, and semi-urbanized zones.

Human activities can contribute to seed dispersal [8,9]; seeds can be spread intentionally (e.g., through sowing and harvesting) or accidentally, for example, via harvesters spreading seeds within and outside fields [10] or through seeds left on roadsides after mowing [11]. Other sources include losses during seeds transportation [12,13] by trains or vehicles [14], among other mechanisms like air movement caused by vehicles [15,16] or even by foot traffic [17,18]. Different studies conducted in various countries indicate that seed loss during transportation by truck or train from cultivation or import areas to processing sites can lead to the establishment of feral oilseed rape populations [19].

The dispersal of GM oilseed rape seeds and the potential establishment of feral populations has been assessed in different countries. A summary of three most relevant case studies conducted in France, Switzerland and Japan, are provided below:

- In France, seed traps were set up along roads from the harvesting fields to the Selommes town grain's stocking site to assess seed loss during transport. The seeds were transported in trucks without tarpaulins, resulting in seed loss. The quantity of dispersed seeds increased with the increase in the surface area of the source field, though it decreased with distance from the fields. Over an eight-day sampling period, an average of 400 seeds per square meters were scattered along the roadside. Genetic analysis linked the dispersed seeds to their field of origin, estimating an average dispersal distance of 1250 m [20,21]. Secondary dispersal was also observed, driven by air turbulence from vehicles, with 20% of the seeds spreading a few meters away and a maximum dispersal distance of 21.5 m [16].
- In Switzerland, despite the prohibition of cultivating and importing GM oilseed rape since 2008, GM oilseed rape plants have been detected along railway tracks and river ports. A study conducted in 2011 and 2012 [22] found glyphosate-resistant GM oilseed rape at several sites along railway, particularly near Ticino and Basel. In Basel, GM populations were discovered at both railway and port locations, including plants resulting from crossings between GM and non-GM varieties were collected but no one resulting from the crossings with interfertile species. The GM populations likely originated from accidental dispersal of seeds during the unloading of Canadian wheat, which contained traces of GM oilseed rape seeds. The persistence of these feral populations seems linked to repeated seed dispersals or herbicide resistance advantages.
- In Japan, although GM oilseed rape is not cultivated, since 2005, GM plants resistant to glufosinate and glyphosate have been detected up to 40 km from major ports, with some of them resulting from the crossbreeding between GM varieties [23,24]. Monitoring over a 10-year period near Kashima port revealed both GM and non-GM feral plants, mostly along roadsides. However, regular road maintenance sharply reduced the number of plants, suggesting that seed dispersal is unlikely to lead to long-term populations if management measures are put in place.

The ERA conducted on the 14 authorized oilseed rape events highlighted the possibility of accidental dispersal of viable seeds, particularly during transportation from the arrival sites of import to the storage sites; other important main environmental exposure is expected to be due to significant losses during the loading or unloading of oilseed rape destined for processing. Such exposure can be controlled through clean-up actions and the application of current practices to avoid the presence of oilseed rape plants, including

manual or mechanical removal and the use of herbicides (except for herbicides whose GM plant is resistant). Importers are therefore required to implement risk management measures, as described above, and establish a surveillance plan to guard against any unforeseen adverse effects on human and animal health or the environment not anticipated by the ERA. To increase the possibilities of detecting unanticipated adverse effects, a monitoring system involving the authorization holder and operators handling viable oilseed rape must be employed.

At a national level, Italy implemented the Directive 2001/18/EC with the Legislative Decree 224 of 8 July 2003, which, in Article 32, requires the adoption of a General Plan for Surveillance Activities (IGPSA). This plan has been adopted by Decree of the Italian Ministry of the Environment of 8 November 2017, and it is implemented by the National Operational Program (NOP), that under Activity II establishes the monitoring of the placing on the market of GMOs as such or contained in products, excluding cultivation. The NOP states: "For GMOs authorized for placing on the market as food and feed pursuant to Regulation (EC) No 1829/2003, the surveillance activity carried out within this National Operational Program aims to verify any environmental effects resulting from the accidental release of GMOs into the environment and the emergence of volunteer plants from viable seeds". In the context of this surveillance activity the Italian National Competent Authority requests to ISPRA and other national technical Institute to define a sampling protocol within storage and handling sites of genetically modified plant material.

To respond the request ISPRA and ARPA Campania have performed a pilot project in Campania region to develop a Monitoring plan for the accidental dispersal of Genetically Modified Oilseed Rape; the results of the project have been used for the development of the sampling protocol of volunteer plants from viable seeds within storage and handling sites (https://bch.mase.gov.it/images/pdf/Protocolli_Campionamento_OGM/Protocollo%20campionamento%20specie%20avventizie%20-%20ottobre%202021.pdf (accessed on 16 April 2025)). Furthermore, the projects' activities led to the definition of a monitoring protocol that complements the developed sampling one and consists of a sampling protocol, transect identification procedures, and DNA analysis. The developed protocol takes into account the Italian territory characteristics. It will be conducted in the hotspots and along transport routes, considering the presence of species interfertile with oilseed rape. The results of the project have been made available to the Italian National Competent Authority and may be applied in the future.

2. Materials and Methods

2.1. Monitoring Plan

The monitoring protocols were developed based on those used in Switzerland [25].

2.2. Mapping and Geolocation

Portable GPS and a web application on Android smartphones were utilized to map, geolocate and register the identified locations:

- Hot spots: Entry points sites, storage sites, and seed sorting centers.
- Major transportation routes: roads connecting hot spots.
- Type 1 transects: Based on risk hypothesis, the sampling stations are within the hot spots, in the adjacent area of 300 m radius and along the entry/exit roads; the latter consist of sections with a length of at least 2 km.
- Type 2 transects: Based on random monitoring, where sampling stations are along transportation routes, comprising 100 m long sections with a width (starting from the road edge) of 2–5 m. Selection of these sections depended on territorial factors, such

as the presence of anthropized or urbanized areas, abundance of species, including interfertile species, for sampling, and feasibility of sampling.

- The number of transects ensured the collection of at least 300 plants per route, which was sufficient to detect the presence of 1% GM oilseed rape with 95% confidence.

2.3. Sampling

Transect Information: During sampling, the following information was recorded: geolocalization, date, time, site description, observations, and a list of interfertile *Brassicaceae* species, including their quantities and assigned sampling codes.

Frequency of inspections: Two inspections per year were scheduled, coinciding with the flowering period of the different Brassica species to be monitored, when potential gene flow by pollen dispersal is most likely to appear.

Sample Collection: Leaves and entire twigs with flowers were collected using scissors or by hand. A sample consisted of 10 individuals of the same species per transect. Each sample was placed in resealable plastic bags, labeled with the assigned code and stored in a portable refrigerator at 20° C below ambient temperature until they were delivered to the laboratory on the same day. In low-density areas ($\leq$30 plants/4 m^2), all individuals, for each species present in the transect, were sampled, while in high-density areas (>30 plants/4 m^2), 20% of individuals were randomly sampled. Samples were then stored in a laboratory freezer at $-80\ ^{\circ}C \pm 5\ ^{\circ}C$.

Bulk Formation: In the laboratory, leaves were separated from stems and frozen, and a bulk sample for subsequent analyses was formed by combining an equal amount of material from each of the 10 individuals of the sample.

2.4. DNA Analysis

DNA Extraction: An aliquot (1–5 g) of the bulk sample was ground into a homogeneous powder using a porcelain capsule with approximately 10 mL of liquid nitrogen. The powder was then transferred into 2 mL Eppendorf tubes. DNA extraction was performed using the Invitrogen Life Technologies "Pure Link Plant Total DNA Purification Kit" with 120 mg of ground sample.

Real-Time PCR: The amplification of the *CruA* gene and screening (p35S, tNOS, construct CTP2/CP4-EPSPS) were conducted using specific primers capable of detecting the presence of transgenes introduced in multiple GM rapeseed events. The reference materials used as positive controls for p35S and tNOS were provided by JRC Directorate F (previous EURL-IRMM) (ERM BF 412d-Bt11 (1%)), while for CTP2/CP4-EPSPS the material used was provided by the company AOCS (AOCS 0304B2 rape GT73/RT73 100%). The standard ISO protocols have been applied: 24276 [26], 21569 [27], 21570 [28].

3. Results

To validate the monitoring and sampling protocols developed by the research group, as outlined in the Section 2, the Campania region was selected as a case study, leveraging the collaboration between ISPRA and ARPA Campania. The activities described below were conducted over the period from 2017 to 2020.

3.1. Identification of Hotspots, Transportation Routes and Transects

As discussed above, in case of the import of GM oilseed rape potential environmental effects can be related to the accidental dispersal of viable seeds during transportation from the arrival sites of import to the storage sites and during loading or unloading in import and storage sites.

In 2017, the pilot project starts with the identification of the main entry points for imported oilseed rape in the Campania region—namely, ports, airports, railway stations,

and storage points—where loading and unloading operations of plant material (seeds or grains) occur, and where the risk of accidental seed loss is the highest. Cost-efficient monitoring can only be conducted if these locations are known.

A list of entities involved in cargo inspections at ports and airports was compiled, including both national institutions, such as the Ministry of Health and the Ministry of Agricultural, Food and Forestry Policies, and regional bodies like the Veterinary Offices for Community Obligations (UVAC), Border Inspection Posts (PIF), and the Experimental, Information, Research, and Agricultural Consulting Sector (SeSIRCA).

Through direct contact with these entities, investigations were initiated to identify entry points and destinations specifically for oilseed rape; destinations included companies working with food and feed, dealing with oilseed processing, or acting as distributors/storage facilities. The findings of this investigation indicate that

- The port of Salerno is the main entry point for oilseed rape plant material.
- There are eight seed companies handling oilseed rape, managing sites where loading/unloading, storage, and processing operations take place.
- Based on the transport system between the point of entry and potential destinations, it became evident that road transport is the most common method, so the monitoring protocol was focused on road routes.
- Since 2016, the import of oilseed rape (*Brassica napus*) to the port of Salerno has significantly decreased, while the import of mustard seeds (*Sinapis alba*) has increased.

To verify the information obtained through direct contact, a field visit was conducted at the port of Salerno to gather data on cargo management and the most commonly used loading/unloading methods for plant material, in order to assess the potential presence and distribution of interfertile species with oilseed rape inside and in the immediate vicinity of the port.

The visit revealed that, while seeds intended for cultivation must be packaged, most of the imported plant material (seeds or grains) destined to feed production arrives and departs in bulks, which significantly increases the risk of accidental dispersion. During the inspection, no oilseed rape plants and its interfertile species were identified within the port's area, while interfertile plant species were collected in areas surrounding the port, confirming the port of Salerno as hotspot.

Since road transport is the primary method for moving goods from the port, railways were excluded from the pathway identification process, and only road routes were considered.

Two main routes were identified for monitoring in this case study:

1. Salerno–Benevento (SA–BN): the port of Salerno as the entry point, with a seed company located in Benevento's municipality as the final destination.
2. Salerno–Caserta (SA–CE): the port of Salerno as the entry point, with Caserta as the final destination, as one of the seed companies is located there, furthermore another seed company is present along the route.

Regarding the monitoring transects, in our specific case, we implemented

- Risk-based monitoring (Type 1): focusing on hotspots such as the port of Salerno and the identified seed companies.
- Random monitoring (Type 2): randomly selecting road sections along the Salerno–Benevento and Salerno–Caserta routes.

3.1.1. Monitoring Activities on the Salerno–Benevento Route

There is a direct highway between Salerno and Benevento, covering approximately 80 km. However, for our purposes, it was necessary to select an alternative route for

two main reasons: first, to find less urbanized areas along the road, such as agricultural, semi-natural, or natural zones; second, to ensure the feasibility of the sampling, which must be carried out under safe conditions for the operators. Thus, we chose a route following state and provincial roads adjacent to the highway, running almost parallel to it, which was considered more relevant for our study.

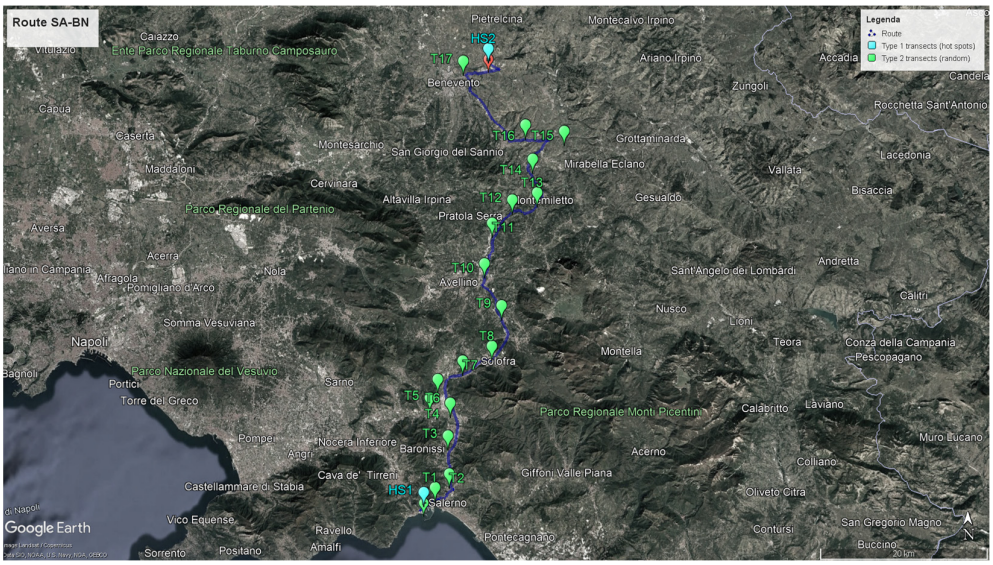
To test the monitoring protocol and, specifically, to verify the feasibility of identifying Type 1 and Type 2 transects as outlined, a site visit was conducted in June 2018 during the flowering period of oilseed rape and other *Brassicaceae* species.

This initial site visit demonstrated that the draft monitoring protocol was generally feasible; however, it was necessary to introduce some modifications to adapt it to the specific territorial and infrastructural characteristics of the study area. Indeed, it was not possible to implement 30 transects, nor was it feasible to maintain a consistent 4 km distance between them, as initially planned, due to limiting factors such as insufficient numbers of plants or unsafe conditions for the operators. Despite the fewer number of transects compared to those planned, we selected areas that allowed for the sampling of the target average number of 10 individuals per transect and the total required number of individuals (300 plants), ensuring the necessary statistical relevance for the monitoring.

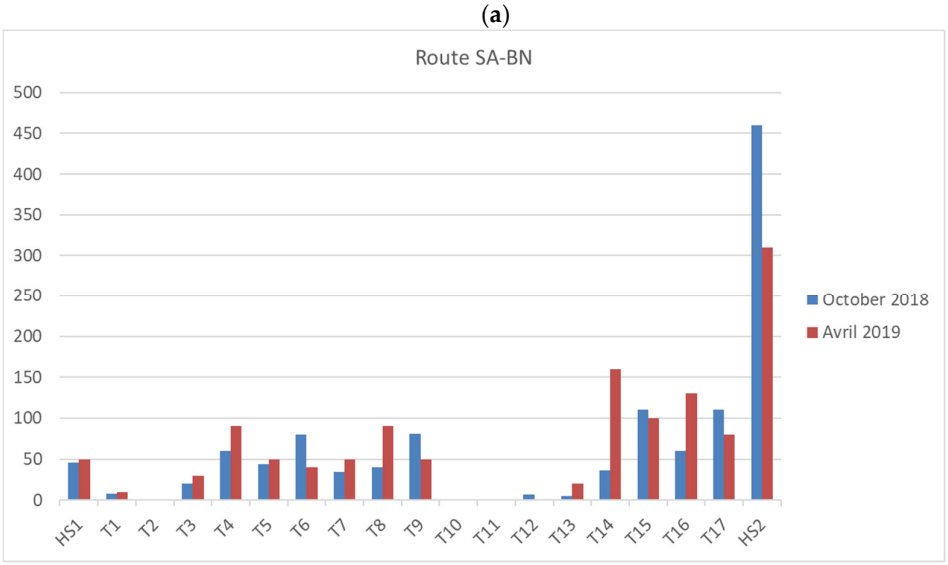
Following the site visit two field surveys were conducted on this route: on October 2018 and May 2019.

The results of the field surveys are as follows (see Figure 1):

- Two hotspots were identified (port and company) to establish Type 1 transects as foreseen by the draft monitoring protocol. The Salerno commercial port is situated on the north border of Salerno municipality. Inside the port clean up measures and the application of practices for the control of adventitious plants are put in place; outside the port area there is a main road used for goods transportation. The port area and its surroundings are characterized by a highly disturbed environment, presenting the typical urban situation: highly anthropized, with small flowerbed areas. As for the second hotspot, it was not possible to enter in the private area of the company, thus the transect has been identified just outside; its environmental characteristics are similar to those found in the surroundings of the Salerno port.
- A total of 17 Type 2 transects were identified; two of these were located near cultivated fields, the rest of them were along roadside verges most of which were not under municipalities cleaning management.
- The average distance between Type 2 transects was approximately 5.5 km, with a maximum distance of 12 km and a minimum of 2.5 km.
- Ten different species of *Brassicaceae* were identified within the transects, including *Brassica napus* L., *Brassica oleracea* L., *Brassica rapa* L., *Diplotaxis tenuifolia* L. DC., *Hirschfeldia incana* L. Lagr.-Foss., *Raphanus raphanistrum* L. (wild), *Raphanus sativus* (domesticated), *Rapistrum rugosum* L. Arcang., *Sinapis alba* L., and *Sinapis arvensis* L.
- A total of 2461 plants were sampled in the two field visits.
- In almost all Type 2 transects there was a low-density situation, with the number of individuals $\leq$ 30 plants per 4 m^2, and all the present individuals were sampled.
- The average number of sampled individuals per Type 2 transects in the two field visits was around 94, from a minimum of 0 to a maximum of 160.
- Near the seed company (Type 1 transect) we found a high-density situation, with a considerable number of individuals of *Raphanus raphanistrum* L. (wild) and *Sinapis alba* L.
- All transects were geolocated using two systems: a handheld GPS and the GPS web application on an Android smartphone.



(**a**)



(**b**)

Figure 1. (**a**) Transects identified along the Salerno–Benevento route during the two field surveys. The blue line represents the path from the starting point (Salerno port) to the destination (the seed company). (**b**) Number of plants collected at each transect along the Salerno–Benevento route during the two field surveys.

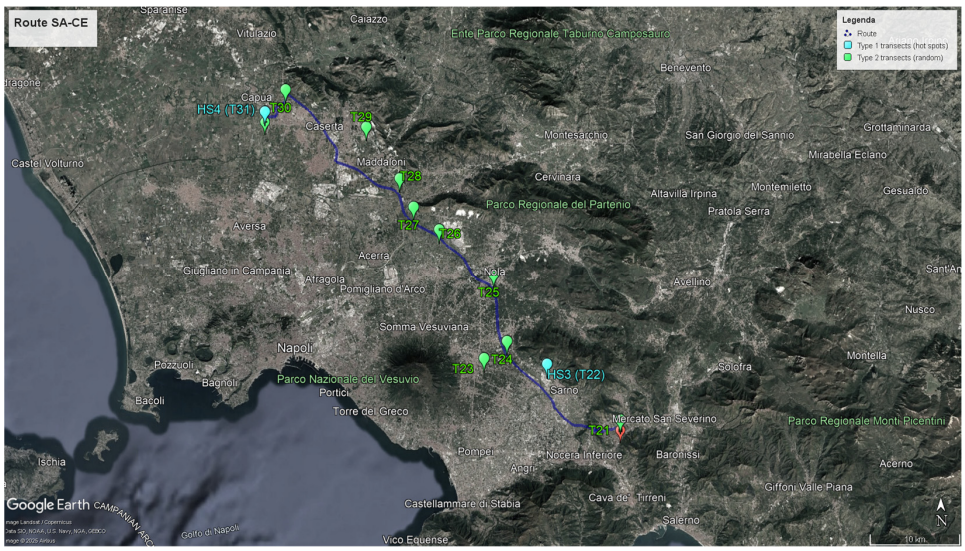
3.1.2. Monitoring Activities on the Salerno–Caserta Route

There is a direct highway between Salerno and Caserta, covering approximately 87 km. As with the previous route, an alternative to the highway was chosen for the same reasons mentioned earlier. Along this route, two campaigns took place in May 2019 and October 2019.

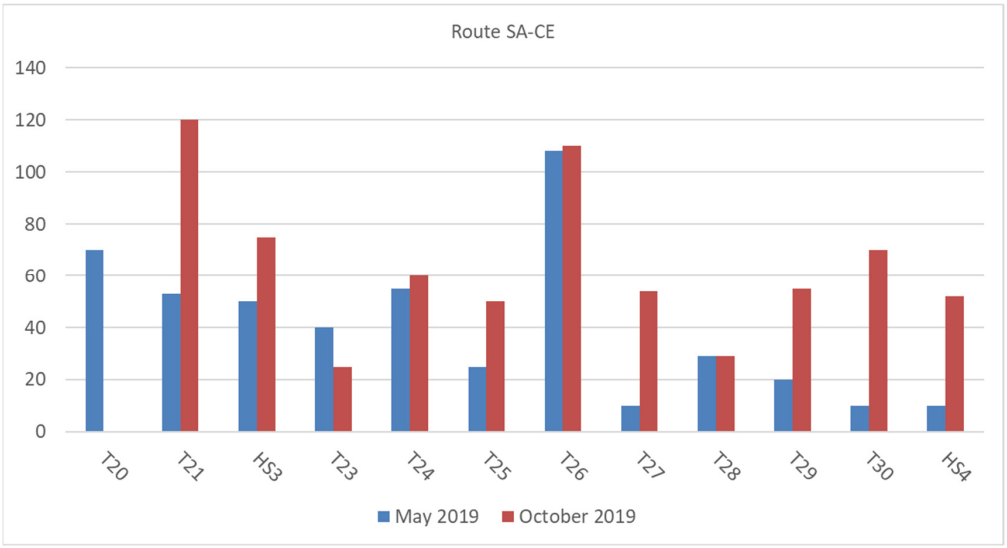
The results of the field surveys are as follows (see Figure 2):

- Two hotspots were identified in two seed companies to establish 2 Type 1 transects (HS3 and HS4) as foreseen by the draft monitoring protocol. The environments in these transects were similar to those found in Salerno-Benevento route.
- A total of 10 Type 2 transects were identified. The environments in these transects were similar to those found in Salerno–Benevento route.
- The average distance between transects was approximately 9 km.
- Fewer species of *Brassicaceae* interfertile with oilseed rape were found compared to the other route (*Brassica napus* L., *Diplotaxis tenuifolia* L. DC., *Raphanus raphanistrum* L.

(wild), *Rapistrum rugosum* L. Arcang., *Sinapis arvensis* L.); in particular on T30, located at the edge of an alfalfa field, only one species was found.
- A total of 1180 plants were sampled in the two field visits.
- In almost all Type 2 transects, there was a low-density situation, with the number of individuals $\leq$ 30 plants per 4 m^2, and all the present individuals were sampled.
- The average number of sampled individuals per Type 2 transect in the two field visits was about 100 (with a minimum of 10 and a maximum of 120).
- Neither of the two seed companies (Type 1 transects) had high-density *Brassicaceae* populations in their surroundings.
- All transects were geolocated using two systems: a handheld GPS and the GPS web application on an Android smartphone.



(**a**)



(**b**)

Figure 2. (**a**) Transects identified along the Salerno–Caserta route during the two field surveys. The starting point for this route was not the port of Salerno, as it had already been considered as starting point for the Salerno–Benevento route, where sampling had been conducted a few weeks prior. Transect T22 was renamed HS3, as it corresponds to a seed company and was classified as a hotspot. Transects T20 and T21, although located at the same site (but on different sides of the road), were screened separately in May 2019. (**b**) Number of plants collected at each transect along the Salerno–Caserta route during the two field surveys.

3.2. Species Interfertile with Brassica napus L. Present in Italy and Selection of Plants Species to Monitor

Oilseed rape (*Brassica napus* L., var. *oleifera*) is a dicotyledon of the *Brassicaceae* family, whose origin can be traced to the Mediterranean basin, the Atlantic coasts of Central Northern Europe, and the northwest of West Africa. Oilseed rape, due to its botanical and agronomic characteristics (see Table 1), is considered a pioneer species with invasive and weedy traits; the numerous seeds produced can disperse over long distances and remain viable for up to 10 years. Moreover, *B. napus* can grow in highly degraded areas such as roadsides, railway embankments, and semi-urbanized zones.

Several species within the *Brassicaceae* family of agronomic and of ecological interest are present in Italy, both in cultivated form and as wild or naturalized populations, often sharing habitats suitable for oilseed rape growth [29]. Twelve of these species can hybridize with *B. napus* (interfertile species) with different degrees of probability (see Table 2). A Canadian monitoring study has demonstrated, following the crossing between GM varieties and non-GM wild or cultivated rapeseed varieties, the transfer of transgenes from GM oilseed rape to related wild plants (e.g., *Raphanus raphanistrum*, *Rapistrum rugosum*, *Hirschfeldia incana*) [30]. One of the objectives of the project was to assess the presence of *B. napus* interfertile species in the identified transects, that are in area/habitats where plants can grow. Record cards of the identified species have been prepared to facilitate their identification; furthermore, the monitoring activities were conducted in months in which it was easier to distinguish the different species (see Table 3).

See Tables 1–3 for further information.

Table 1. Summary of the main characteristics of oilseed rape (*Brassica napus* L.).

Cultivation in Italy	Reproduction and Survival	Ecology
Sowing Period: August-November (in Northern Italy, second half of September; in Central/Southern Italy, sowing is also possible in October-November) Flowering: March-October Maturation: March-October Harvest: March-October	Oilseed rape reproduces by seed and can spread at the edges of crops, where it can hybridize with other species of the Brassica genus, both cultivated and wild. The seeds can remain viable up to 10 years	It is a naturalized archaeophyte in the regions of Piedmont, Lombardy, Veneto, Friuli Venezia Giulia, Tuscany, Lazio, Molise, and Sardinia, and it is a casual alien in other regions. It is interfertile with various *Brassicaceae* species.

Table 2. Common species interfertile with *Brassica napus* L. in Italy and their reference habitats.

Species	Habitat	Probability of Hybridization *	Literature Reference
Brassica juncea (L.) Czern.	Margins of crops, ruderal environments	1	[23,31]
Brassica nigra (L.) Koch	Cultivated	1	[29,32]
Brassica oleracea L.	Margins of crops, cliffs, urban green areas	3	[33,34]
Brassica rapa L.	Margins of crops	1	[7,29,32,34–37]

Table 2. *Cont.*

Species	Habitat	Probability of Hybridization *	Literature Reference
Diplotaxis muralis (L.) DC.	Arid Mediterranean pastures	4	[38]
Diplotaxis tenuifolia L. DC.	Margins of crops, abandoned agricultural areas, road edges, fallow agricultural fields, urban green areas	4	[39]
Hirschfeldia incana L.	Margins of extensive crops, abandoned agricultural areas, Mediterranean and sub-Mediterranean pastures, olive groves	2	[29]
Raphanus raphanistrum L. subsp. *raphanistrum*	Margins of crops, abandoned or fallow agricultural fields, road edges, nitrophilous and sub-nitrophilous meadows, olive groves	2	[29,31,37,40–42]
Raphanus sativus L.	Cultivated	4	[34,42,43]
Rapistrum rugosum L.	Margins of herbaceous crops, gardens, and olive groves, sub-nitrophilous meadows	4	[37]
Sinapis alba L.	Margins of extensive crops, gardens, abandoned or fallow agricultural fields, road edges	4	[36,37]
Sinapis arvensis L.	Margins of extensive crops, abandoned or fallow agricultural fields, road edges, urban green areas	4	[29,32,36,37];

* 1 = high probability (SH, F1, F2, BcP); 2 = medium-high probability (SH, SH(BnMS), F1, BcP); 3 = low probability (F1, F2, BcP); 4 = rare event (F1, BcP; F1). SH = spontaneous hybrids formed without the aid of emasculation and manual pollination transfer. SH(BnMS) = spontaneous hybrids with male sterile *B. napus* as female parent. F1 = F1 hybrids produced through intervention of some sort, i.e., emasculation and manual pollination. F2 = F2 hybrids produced. BcP = backcross progeny produced.

Table 3. Visual comparison between *Brassica napus* L. and other interfertile *Brassicaceae* species present in Italy, regarding the main plant structures (all images taken from the Italian Flora Portal, https://dryades.units.it/floritaly/index.php (accessed on 16 April 2025)) and indication of geographic presence.

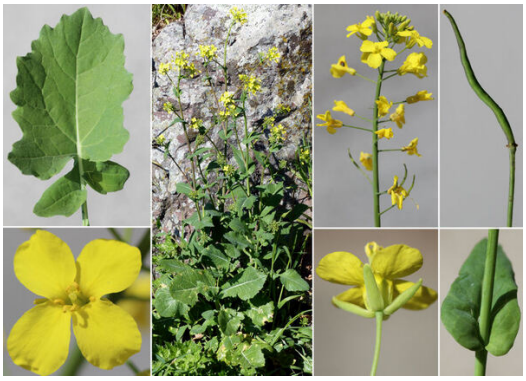
Species	Flower	Presence *
Brassica napus L.		

Table 3. *Cont.*

Species	Flower	Presence *
Brassica juncea (L.) Czern.		
Brassica nigra (L.) Koch		
Brassica oleracea L.		

Table 3. *Cont.*

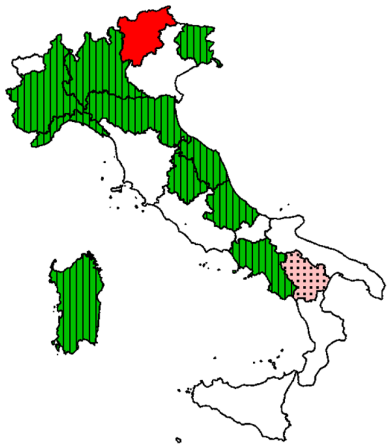
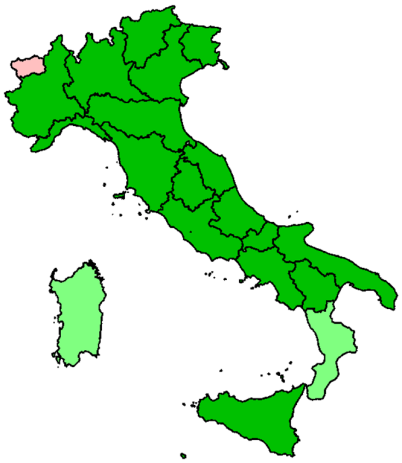
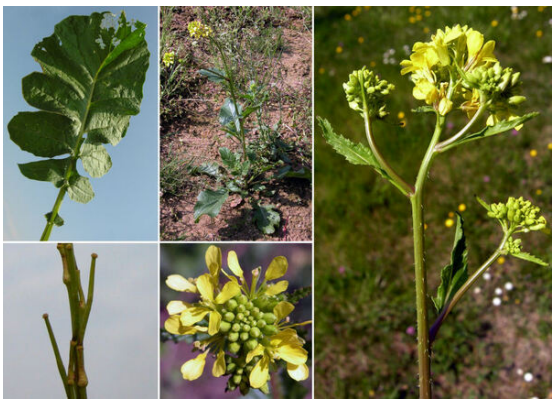
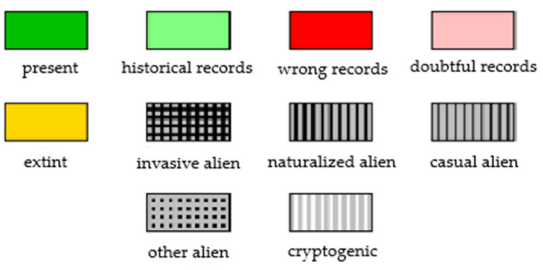
Species	Flower	Presence *
Brassica rapa L.		
Diplotaxis muralis (L.) DC.		
Diplotaxis tenuifolia L. DC.		

Table 3. *Cont.*

Species	Flower	Presence *
Hirschfeldia incana L.		
Raphanus raphanistrum L. subsp. *raphanistrum*		
Raphanus sativus L.		

Table 3. *Cont.*

Species	Flower	Presence *
Rapistrum rugosum L.		
Sinapis alba L.		
Sinapis arvensis L.		



*

3.3. Monitoring of Interfertile Species of Oilseed Rape Presence in Campania

The results of the sampling activities performed between October 2018 and October 2019, along the two selected routes, show that, of the most interfertile species present in Italy, the only one actually present and in significant quantities in all the identified transects, is the wild radish (*Raphanus raphanistrum*). Figure 3 below presents a summary of the sampling activities.

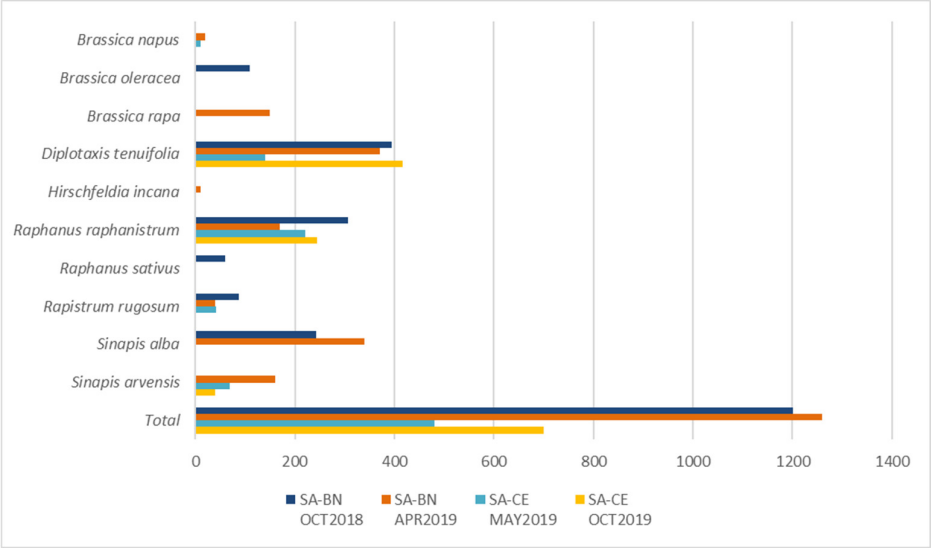
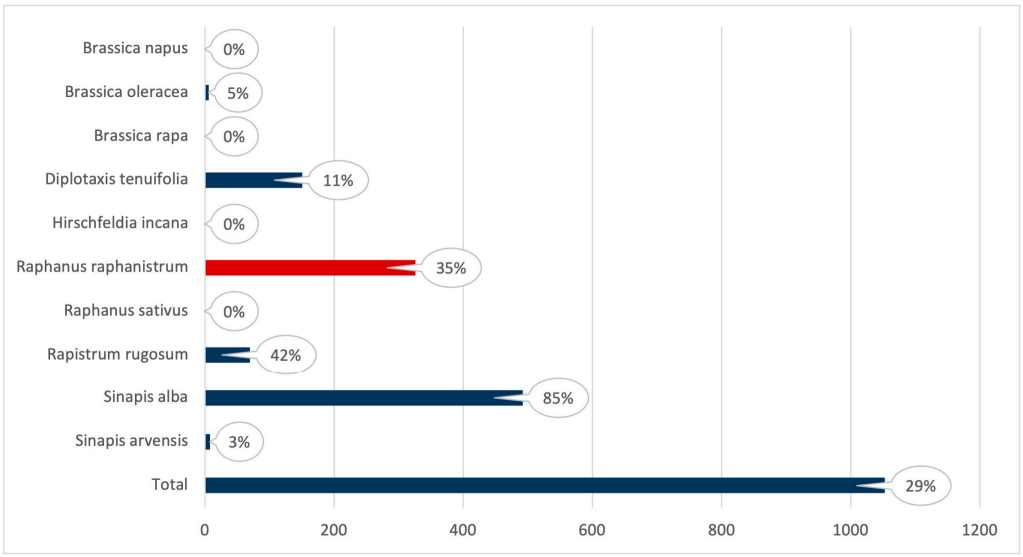


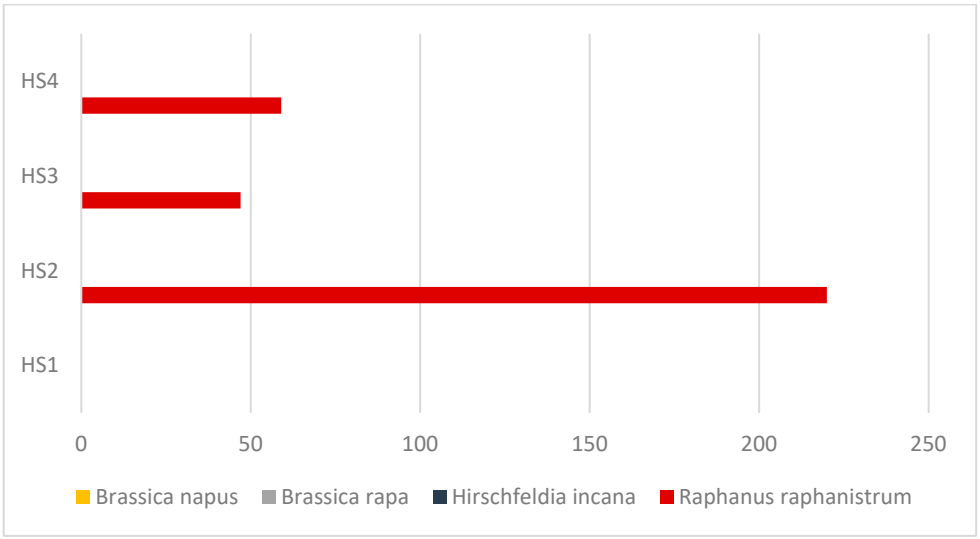
Figure 3. Individuals sampled along the two routes during the four sampling sessions conducted between October 2018 and October 2019.

As shown in Figure 4, wild radish is present near the three seed companies (HS2, HS3, HS4). A special note should be made regarding the hot spot at the port of Salerno (HS1): only a few individuals of a few species were found inside and near the port, likely due to the fact that it is subject to plant health treatments and that the immediate surroundings are highly urbanized (there are only some flower beds present).



(**a**)

Figure 4. *Cont.*



(**b**)

Figure 4. (**a**) Presence of species interfertile with oilseed rape in the four hot spots identified along the two routes (values reported as a % of the total individuals sampled in the four field surveys). (**b**) Presence of species with the highest degree of hybridization with oilseed rape (1 and 2, according to Table 2 above) in the four hot spots identified along the two study routes.

3.4. Samples Analysis

After each sampling campaign, the collected samples were analyzed by the Regional GMO laboratory of Campania region, the laboratory is part of the Italian Network of GMO Laboratories (NILO).

All collected 367 bulks, corresponding to 3641 individuals, were analyzed following the procedures described in the Section 2. All samples contained high-quality DNA free from PCR inhibitors, as demonstrated by the consistency of ΔCt values with the expected dilution factors and the absence of amplification in the negative controls.

These results confirm the reliability of the DNA extraction method, and the RT-PCR protocol used for the detection of the endogenous *CruA* gene (a genetic marker for *Brassicaceae*), including the positive control GM oilseed rape and bulk samples of *Brassicaceae* collected during the field surveys (see Table 4).

Table 4. Number of bulks collected and analyzed by species. SA–BN OCT 2018, SA–BN APR 2019, SA–CE MAY 2019, and SA–CE OCT 2019 correspond to (a) the Salerno–Benevento route, October 2018; (b) the Salerno–Benevento route, April 2019; (c) the Salerno–Caserta route, May 2019; and (d) the Salerno–Caserta route, October 2019.

	SA–BN OCT2 018	**SA–BN APR 2019**	**SA–CE MAY 2019**	**SA–CE OCT 2019**	**Total**
Brassica napus	2	0	1	0	3
Brassica oleracea	0	13	0	0	13
Brassica rapa	15	0	2	0	17
Diplotaxis tenuifolia	26	41	16	44	127
Hirschfeldia incana	1	0	0	0	1
Raphanus raphanistrum	15	31	24	24	94
Raphanus sativus	0	6	0	0	6
Rapistrum rugosus	2	9	5	0	16
Sinapis alba	34	25	0	0	59
Sinapis arvensis	16	0	10	5	31
Total	111	125	58	73	367

All the samples positive for the endogenous *CruA* gene were screened by Real-Time PCR targeting the 35S promoter, NOS terminator, and ctp2/CP4-epsps insert. These sequences were selected because, in 2020, GM oilseed rape events authorized or under authorization in the EU, carried at least one of them. No amplification was observed in any sample, indicating the absence of detectable GM material.

4. Discussion and Conclusions

Currently, 14 genetically modified (GM) oilseed rape events, including staking events, are authorized for commercialization in Europe: none are authorized for cultivation, and most of them contain an herbicide-resistant (HR) gene. Even if cultivation does not occur, the accidental spillage of seeds destinated to food or feed production may occur. The dispersed seeds can give rise to small populations of feral GM oilseed rape that can survive and propagate thanks to the pioneering and invasive characteristic of the species. The crossing of the GM population with the interfertile species is a plausible scenario that has been assessed in the environmental risk assessment (ERA) performed for the authorized events. Furthermore, the herbicide-resistance characteristic may provide a selective advantage in cultivated fields and on roadsides; indeed, glyphosate-based, glufosinate-ammonium or dicamba-based herbicides are commonly used for the management of invasive or weed. Herbicide resistant plants surviving to the herbicide treatments may lead to the progressive expansion of spontaneous populations, both of the GM oilseed rape itself and of the resulting crossing. Although these risks are considered remote, the need for specific management and monitoring measures has been acknowledged. These measures require general surveillance, which should be proportionate to the extent of oilseed rape imports and implemented for the duration of the authorization.

In Europe, the presence of feral GM oilseed rape has been detected in several instances, particularly near processing facilities and along transport routes (e.g., Switzerland, France, etc.). Switzerland has implemented a monitoring plan to identify GM oilseed rape along its transport routes [25], revealing systematic findings of oilseed rape in these areas.

Since there is a strong presence of both cultivated oilseed rape and wild interfertile species in Italy, as shown in Table 2, it is particularly important to monitor the presence of GM oilseed rape. Where the presence of a population of GM oilseed rape is detected, it will also be necessary to monitor the presence of species with a high rate of interfertility with oilseed rape, to verify the actual exposure and partially quantify the likelihood of a negative environmental effects following the accidental dispersion of GM oilseed rape seeds. This activity shall be conducted at points where the probability of dispersion is the highest (hotspots).

For these reasons, we have developed a monitoring protocol to assess the accidental dispersion of oilseed rape seeds in Italy, and we tested it on field with a pilot case in the Campania Region. By checking the data on oilseed rape imports in this region, we identified the port of Salerno as the entry point and several seed companies that import oilseed rape as arrival points (hotspots). Since the transport of this material occurs by road, we identified potential routes where we applied the monitoring protocol.

According to the information collected, oilseed rape was imported at the port of Salerno in different years. However, in the years immediately preceding the survey, the movement of oilseed rape at the port of Salerno had significantly decreased. In contrast, the movement of white mustard (*Sinapis alba*) seeds at the seed company near Benevento (HS2) has been reported. Notably, numerous *Sinapis alba* plants were present in the vicinity of seed companies during both years of sampling (see Supplementary Materials). These data support the initial hypothesis that seed companies could serve as hotspots for accidental dispersion. Additionally, it is important to highlight the simultaneous presence, in the

same hotspot and in substantial quantities, of one of the four species with the highest hybridization rates with canola—wild radish (*Raphanus raphanistrum*). The co-occurrence of *Sinapis alba*, used as a proxy of the GM oilseed rape, and an interfertile species supports the hypothesis that gene flow between species may occur. This process could be enhanced by positive selection pressure due to the use of herbicides, of which GM plants are resistant.

The analysis protocol developed by the laboratory in Avellino, the regional reference center for GMO analyses, has also proven to be effective. All the 10 analyzed species belonging to the *Brassicaceae* family tested positive for the endogenous *CruA* gene, confirming the reliability of both the sampling (including procedures related to sample collection, bulk formation and sample conservation until arrival at the analytical laboratory) and analysis procedures. Furthermore, as also highlighted by the Joint Research Centre [44], GMO-screening strategies should be regularly revised in accordance with the evolving list of authorized GMOs in the European Union.

The developed monitoring protocol has proven to be valid, although adjustments were necessary following field inspections. It is therefore suggested to always conduct field visits to assess the local feasibility of the protocols and modify them based on specific conditions of the territory and infrastructures. It is especially important to verify the possibility of maintaining the distance specified in the monitoring protocol and the presence of a sufficient number of individuals to ensure the statistical relevance of the sampling.

Under EU law, Post-Market Environmental Monitoring (PMEM) is the responsibility of the marketing authorization holder for genetically modified (GM) oilseed rape, represented by EuropaBio. EuropaBio is required to ensure that the monitoring plan is implemented in accordance with authorization conditions. This plan mandates that member companies establish procedures (e.g., ISO, HACCP) to minimize seed loss, eradicate adventitious populations, and manage herbicide-resistant (HR) populations as potential adverse effects. Additionally, companies must report any adverse effects, including those occurring during seed transportation, to European trade organizations.

However, several documented cases have demonstrated that these measures are insufficient to fully prevent the accidental spillage of GM seeds. The presence of HR genes provides GM oilseed rape with a selective advantage in areas where herbicides are used for the management of ruderal zones, potentially leading to the establishment of feral populations.

To address these concerns, we recommend strengthening PMEM by identifying high-risk areas for accidental GM seed release, such as entry ports, industrial sites where GM seeds are stored or processed, and transportation corridors, including railways, waterways, and roads. Additionally, PMEM can be reinforced through targeted initiatives by member states. The findings of our research project confirm that accidental seed dispersal can occur. The protocols, for sampling and DNA analysis, developed and tested in the pilot study in Campania can serve as a useful framework to address PMEM requirements outlined in the Directive. Indeed, the sampling protocol of volunteer species within storage and handling sites has been adopted by the Ministry of Environment and Energy Security to support the implementation of the Activity II of the Official General Plan for Surveillance on the deliberate release of Genetically Modified Organisms into the environment foreseen in the Ministerial Decree of 8 November 2017. Furthermore, stricter measures should be implemented to minimize seed dispersion during transport, unloading, storage, and handling of GM seeds to reduce the risk of unintended environmental exposure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/seeds4020020/s1, Supplementary Materials-Interfertile species collected across transects.

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