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RESEARCH ARTICLE

Transgenic glyphosate-resistant oilseed rape (*Brassica napus*) as an invasive weed in Argentina: detection, characterization, and control alternatives

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Abstract The presence of glyphosate-resistant oilseed rape populations in Argentina was detected and characterized. The resistant plants were found as weeds in RR soybeans and other fields. The immunological and molecular analysis showed that the accessions presented the GT73 transgenic event. The origin of this event was uncertain, as the cultivation of transgenic oilseed rape cultivars is prohibited in Argentina. This finding might suggest that glyphosate resistance could come from unauthorized transgenic oilseed rape crops cultivated in the country or as seed contaminants in imported oilseed rape cultivars or other seed imports. Experimentation showed that there are alternative herbicides for controlling resistant *Brassica napus* populations in various situations and crops. AHAS-inhibiting herbicides (imazethapyr, chlorimuron and diclosulam), glufosinate, 2,4-D, fluroxypyr and saflufenacil proved to be very effective in controlling these plants. Herbicides evaluated in this research were employed by farmers in one of the fields invaded with this biotype and monitoring of this field showed no evidence of its presence in the following years.

Keywords *Brassica napus* · Transgene escape · Glyphosate · GMO · Seed contaminants · GT73 · Invasiveness

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Introduction

Oilseed rape (*Brassica napus* L.) is one of the most important sources of vegetable oil, and it is grown on approximately 36 Mha globally. In 2015, 24 % of the area dedicated to oilseed rape was cropped with genetically modified (GM) cultivars. The main commercial trait is herbicide resistance (HR), and five countries currently grow GM HR oilseed rape: Canada, USA, Australia, Japan, and Chile (FAOSTAT 2016; International Service for the Acquisition of Agri-Biotech Applications (ISAAA) 2016).

Three types of herbicide-resistant transgenic oilseed rape have been approved in some of these countries: glyphosate-, glufosinate-, and bromoxynil-resistant cultivars (International Service for the Acquisition of Agri-Biotech Applications (ISAAA) 2016). Glyphosate resistance (event GT73) is conferred by a single genetic construct containing two genes: one encoding a herbicide-insensitive 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4 (CP4 EPSPS), the target enzyme for glyphosate, and the other a *gox* gene (*goxv247*) that encodes glyphosate oxidoreductase, an enzyme that degrades glyphosate (Green 2009). These cultivars are commercialized under the trade name Roundup-Ready™ and represent the largest proportion of the GM oilseed market worldwide. Glufosinate-resistant cultivars include the *pat* or *bar* gene from *Streptomyces* bacterium, which encodes for the enzyme phosphinothricine acetyl transferase (PAT) that detoxifies glufosinate by acetylation. GM

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bromoxinyl-resistant oilseed rape has been produced using the *oxy* gene from *Klebsiella ozaenae* that encodes a nitrilase enzyme responsible for the breakdown of bromoxinil to a non-phytotoxic compound. There are also non-GM cultivars with herbicide resistance to AHAS-inhibiting (obtained by mutagenesis and traded as Clearfield® oilseed rape) and triazine herbicides (natural mutation and backcrosses) (McVetty and Zelmer 2007).

B. napus is an amphidiploid, most likely originating from multiple spontaneous interspecific hybridizations between turnip rape (*Brassica rapa*; AA, $2n = 2X = 20$) and cabbage or kale (*Brassica oleracea*; CC, $2n = 2X = 18$). The oil form of *B. napus* does not occur in the wild (Allender and King 2010; Prakash et al. 2012). Extensive domestication of *B. napus* is relatively recent, compared with highly domesticated cereal crops, and oilseed rape may still retain more weedy characteristics than other crops. This includes rapid growth development and reproductive maturation, high individual plasticity, high reproductive capacity, and seed dispersion (Hall et al. 2005).

Due to its significant dehiscence, a considerable amount of seed becomes incorporated into the soil seed bank, prior to or during oilseed rape harvest (Gulden et al. 2003). *B. napus* seeds are smaller than the seeds of most other crops, smooth and spherical, facilitating self-burial (Gulden et al. 2008). Although primary dormancy is not found in oilseed rape, stress conditions may result in the development of secondary seed dormancy, allowing the survival and perpetuation of individuals (Pekrun et al. 1997; Gulden et al. 2004). It has been confirmed that secondary dormant seed may persist for up to 10 years or more under field conditions (Lutman et al. 2003). For instance, 10 years after a trial of GM herbicide-resistant oilseed rape in Sweden, GM emergent seedlings were found (D'Hertefeldt et al. 2008). In organic fields from Denmark, plants of oilseed rape varieties cultivated 8–11 years previously were detected (Andersen et al. 2010).

The offspring of oilseed rape seed that shattered prior or during harvest can create weed problems in future crops. These plants living within agricultural fields as a result of previous cropping are known as volunteers (Gressel 2005). Volunteer *B. napus* is particularly common in oilseed rape-growing areas of the USA and western Canada where it was ranked as the 10th most common weed in the mid-1990s (Simard et al. 2002; Beckie et al. 2006; Gulden et al. 2008). Volunteer *B. napus* is another weed problem in several countries of Europe (Andersen et al. 2010; Krato and Petersen 2012; Kolářová et al. 2013; Weber et al. 2014).

Under some conditions, oilseed rape has the ability to escape from cultivation and grow in natural and seminatural habitats, forming persistent populations (Devos et al. 2012). These populations, which can survive and reproduce successfully without any human intervention, are known as feral populations (Gressel 2005).

Feral oilseed rape has been reported in several regions where the crop is grown widely, e.g., Canada, the USA, Australia, and the EU (France, UK, Germany, and Denmark). It often occurs in ruderal habitats such as field margins, road verges, railway lines, ports, and seed storage and manipulation facilities (Pivard et al. 2008a; Elling et al. 2009; Pascher et al. 2010; Squire et al. 2011; Devos et al. 2012; Busi and Powles 2016). Feral oilseed rape populations may originate due to the spillage of seed during transportation, the redistribution of seed by field equipment, or its dispersal by birds and mammals (von der Lippe and Kowarik 2007; Bailleul et al. 2012; Devos et al. 2012).

The dynamics of feral populations at one location is mostly dependent on seed immigration from both agricultural fields and transport (as fresh seed spills). However, it has been shown that soil seedbanks and local recruitment from seed produced by resident ferals can be an important source contributing to population persistence (Pivard et al. 2008a). The persistence of varieties that are no longer grown or commercialized was detected in France (Pessel et al. 2001), Germany (Elling et al. 2009), Austria (Pascher et al. 2010), and Denmark (Pascher et al. 2010).

In countries where GM HR oilseed rape is commercially grown such as Canada and the USA, the occurrence of feral GM HR oilseed rape plants has been confirmed, along the margins of agricultural fields, as well as along transportation routes (Yoshimura et al. 2006; Knispel et al. 2008; Schafer et al. 2011; Devos et al. 2012).

In Japan, where cultivation of GM oilseed rape was banned but it was imported for food and feed, feral glyphosate- and glufosinate-resistant GM oilseed rape plants have repeatedly been detected in port areas and along transportation routes. Transgene presence was attributed to the accidental loss and spillage of imported viable GM HR oilseed rape seed (Saji et al. 2005). The persistence of these populations was recorded for over 6 years, with no increase or decrease in their density, showing no invasive aptitude in ruderal habitats (Katsuta et al. 2015; Nishizawa et al. 2016).

In Switzerland, where GM oilseed rape has not been cultivated or imported, the occurrence of glyphosate- and glufosinate-resistant oilseed rape has been reported. The occurrence of GM oilseed rape was attributed to spillage of conventional OSR seeds or other seed imports that were contaminated with GM seeds (Schoenenberger and D'Andrea 2012; Hecht et al. 2014; Schulze et al. 2014).

The production of oilseed rape in Argentina is limited, with a planted area of around 36,000 ha in the last decade and a production of 59,000 t. It represents a minor oilseed crop, compared with soybean (17.7 Mha, 47.1 Mt) and sunflower (1.8 Mha, 3.2 Mt) or with cereal crops like wheat (4.3 Mha, 12.1 Mt) and maize (3.6 Mha, 23.8 Mt). The importation of oilseed rape seed in the country is scarce (<30 t/year in the last decade) (FAOSTAT 2016; Ministerio de Agroindustria

(MinAgro) 2016). Presently, five seed companies offer about 24 cultivars of oilseed rape and all of them are introductions made from the principal breeding centers of the world. The main countries of provenance of the marketed seed are the USA, Canada, Australia, Germany, and Sweden (Iriarte 2015; Instituto Nacional de Semillas (INASE) 2016). GM oilseed rape has never been cultivated in Argentina, and its cultivation has been banned since 1997, when the national Secretariat of Agriculture forbade experimental production of GT73 GM oilseed rape (Secretaría de Agricultura, Ganadería, Pesca y Alimentación (SAGPyA) 1997). In 2007, the ban was extended and all import of GM oilseed rape for production or commercialization was forbidden and the reception and processing of any import of oilseed rape seed required the presentation of a GMO free analysis from the exporting country (Secretaría Nacional de Sanidad Ambiental (SENASA) 2007). There have been no records of experimental evaluations in field trials of GM oilseed rape in the country (Comisión Nacional Asesora de Biotecnología Agropecuaria (CONABIA) 2016).

However, in 2012, some agricultural fields with no recent records of oilseed rape cultivation were invaded with plants of *B. napus* that could not be controlled with glyphosate applications. The aim of this study was to characterize the herbicide resistance profile of these uncontrolled weedy oilseed rape populations and to determinate the origin of this resistance.

Materials and methods

Plant material and growth conditions

During the spring of 2012, the mature pods from a minimum of 15 plants of three *B. napus* accessions (GER, SMA, and REG) were collected. These plants had survived glyphosate application on three different farms in the southeast of Buenos Aires province and were reported to technical personnel of BASF Co. by the farmers. The populations formed large groups inside RR soybean crops and fallow lots, on farms with no recent records of oilseed rape cultivation. A conventional cultivar of oilseed rape (BNC) was used for comparison purposes, as a negative control ("Nexera 1700," Dow Agrosciences Co.).

During the collection, the flora community composition was observed, with special emphasis in Brassicaceae weeds and oilseed rape wild relatives. Historical records of the fields were provided by the technical managers of the farms to evaluate the possible origin of *B. napus* populations and the cultivation of oilseed rape crops in the past.

For all assays, plants were established by sowing ten seeds in 15-cm-diameter plastic pots containing 75 % soil and 25 % potting mix (Grow Mix Terrafertil, with composted bark, peat moss, vermiculite, calcite, and dolomite). Plants were grown in the greenhouse at 20 ± 5 °C, watered twice daily and fertilized with a liquid fertilizer (Chase LI, grade 5-3-3).

Morphological and chemical characterization

In order to correctly identify the species of the plants found in the fields, and detect any possible segregation due to hybridization with wild relatives, the progeny of the GER *B. napus* population was grown in a common garden at the Agronomy Department, Universidad Nacional del Sur, Bahía Blanca, Argentina (S 38° 41′ 38″, W 62° 14′ 53″) during the 2013 and 2014 seasons. Three *B. napus* accessions (BNC, BNR, and GVI) and six *B. rapa* natural populations (BAL, QUE, ENE, SDV, JUA, and SCB) were used as controls, as this species is the closest wild relative and an important weed in Argentina, which share similar morphological characteristics (Gulden et al. 2008). Seedlings were grown in 28 × 54 cm 200-cell plastic trays containing commercial substrate in the greenhouse under natural light at 20–25 °C for 30 days and then transplanted to the field. Accessions were cultivated in the experimental field for 2 years under a planting pattern of 0.3 × 0.5 m and with drip irrigation for optimal plant growth.

Phenotypic characterization was based on 30 descriptors, 15 quantitative and 15 qualitative characters from the International Board for Plant Genetic Resources *Brassica* L. and *Raphanus* L. descriptors list (International Board for Plant Genetic Resources (IBPGR) 1990). These characters were used as they have taxonomic value for differentiating both species correctly (Mulligan 1995; Gulden et al. 2008). Quantitative traits of the individual plants included: cotyledon width (cm), mature plant height (cm), number of branches (*n*), leaf width (cm) and length (cm), leaf size (cm^2), and petiole length (cm) measured on the lower leaves at the flowering stage; on the main inflorescence: petal length (cm) and number of siliques (*n*). Quantitative traits measured on 20 siliques per accession included silique length (cm) and width (cm), beak length (cm), beak/valve ratio, number of seeds per silique (*n*), and average fresh seed weight (mg).

The qualitative traits included the leaf color, pubescence, shape, margin, and incisions in the upper and lower leaves and also color of petals, disposition of open flowers in respect to the buds and the disposition of the siliques observed on the main inflorescence. Categorical root traits—form and color—were also measured. The life cycle was computed for each population as days from transplant to the beginning of flowering (50 % of plants in bloom) and total cycle duration (transplant to 90 % of fully dry plants).

In order to determine whether the *B. napus* populations could be identified as canola cultivars—defined as seed, oil, and meal that contain <1 % of the total fatty acid as erucic acid and <18 mmol of aliphatic glucosinolates (Prakash et al. 2012)—the oil concentration (%) and fatty acid composition were analyzed by nuclear magnetic resonance and gas

chromatography. Grain samples (50 g) of GER, six oilseed rape accessions (BNR, GVI, DOR, BNN, MCH, and BAL-N), and six *B. rapa* natural populations (BAL, ENE, QUE, SDV, SUA, and BAR) were used. The analyzed fatty acids included palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidic (C20:0), eicosenoic (C20:1), behenic (C22:0), and erucic (C22:1) acid content (%) and the glucosinolate content (μg).

ANOVA of quantitative data were analyzed as an incomplete block design, considering the year as a block, and the accession as the main factor. Qualitative data were analyzed using the non-parametric Kruskal-Wallis test. Quantitative data were analyzed by principal component analysis (PCA) to explore differences between accessions, with traits grouped in morphology, oil concentration, and composition. Analyses were performed using the statistical software Infostat (Di Rienzo et al. 2015).

Herbicide screening test

The response of the GER, SMA and REG *B. napus* accessions to glyphosate was determined. Glyphosate (Glifoglex 48 %, 356 g a.e. L^{-1}, Gleba S.A., Melchor Romero, Bs. As., Argentina) was applied at double the recommended rate (X = 1.29 kg a.e. ha^{-1}) 42 days after emergence (DAE) when plants had three to four leaves, using a conveyor belt carrying the plants under a stationary sprayer equipped with flat spray tips (TeeJet 8001 EVB), at 1.54 km h^{-1} and calibrated to deliver 127 L ha^{-1}.

Plant response was evaluated 35 days after treatment (DAT). Plants were classified as herbicide survivors if the growing point remained alive. Survival was assessed using a visual scale that classified individual damage in the following categories: 1 = no damage, 2 = ≤25 % leaf damage, 3 = 26–75 % leaf damage, 4 = >75 % leaf damage, and 5 = dead plant. Biotypes were considered resistant if 20 % of the individuals survived the recommended herbicide rate for field application. Above-ground plant tissue was harvested at 40 DAT and dried at 50 °C until constant weight to obtain the dry matter value.

The experiment was arranged as a completely randomized design, with four replications. Data was transformed by:

$$y = \arcsin(x + 0.5)^{1/2} \quad (1)$$

ANOVA analysis and a mean comparison Tukey's test were conducted with R3.0.2 statistical software (R Core Team 2015).

Dose-response assay

A dose-response experiment on the GER glyphosate-resistant accession was conducted with herbicide at 0-, 1/10-, 1/5-, 1/2-, 1-, 2-, 5-, 10-, 15-, and 30-fold the commercial field rate. The susceptible population was treated with 0-, 1/50-, 1/20-, 1/10-, 1/5-, 1/2-, 1-, 2-, 5-, and 10-fold the commercial field rate. Dose-response experiments were repeated in two different years.

Herbicide was applied at 29 DAE using a conveyor belt carrying the plants under a stationary sprayer equipped with flat spray tips (TeeJet 8001 EVB) at 1.54 km h^{-1} and calibrated to deliver 175 L ha^{-1}.

Plant survival was evaluated at 35 DAT and dry matter obtained at 40 DAT. Data was fitted to a non-linear log-logistic regression model with three parameters. Dose-response curves were made using the drc package of the R3.0.2 statistical software. The effective dose required for 50 % plant injury (ED_{50}) was estimated. This value was used to calculate the resistance factor (RF), defined as the ratio between ED_{50} of the resistant and susceptible biotypes (ED_{50} R/ED_{50} S).

The log-logistic model equation used was:

$$Y = \frac{d}{1 + \exp(b(\log(x) - \log(e)))} \quad (2)$$

where Y is the percentage survival, e is the inflection point of the curve, d is the coefficient corresponding to the upper curve limit, b is the response line slope around e, and x (independent variable) is the herbicide dose. The lower limit was fixed at 0 (three parameters) (Ritz 2010).

Transgene detection

For immunochemical chromatography, 50 to 100 μg of leaf tissue or seeds of the *B. napus* accessions were homogenized with a pestle in 7 mL of the extraction buffer, and the crude extracts were analyzed to detect the enzyme that confers resistance glyphosate (CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase), using the QuickStix strips (EnviroLogix, Portland, ME, USA).

For DNA analyses, DNA was extracted from a leaf of the herbicide-resistant plants using a quick method (Doyle and Doyle 1987) and analyzed by means of PCR, using the primers 5′-CCATATTGACCATCATACTCATTGCT-3′ and 5′-GCTTATACGAAGGCAAGAAAAGGA-3′ for the CP4 EPSPS gene that encodes EPSPS (Monsanto Biotechnology Regulatory Sciences 2004; Mazzara et al. 2007). Amplifications using 60 ng of extracted DNA were carried out in 20 μL reaction mixtures containing 1 × PCR buffer (Gibco), 2.5 mM $MgCl_2$, 0.125 mM of each dNTP, 5.5 pM of each primer, and 1 U of Taq polymerase, with denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 53 °C, elongation at 72 °C for 30 s, and final elongation at 72 °C for 10 min. The PCR-amplified products were then subjected to electrophoresis on 3 % agarose gel.

Alternative herbicides

The susceptibility of the GER accession to nine alternative herbicides for different crops was evaluated (Table 1). Herbicides were applied at the recommended rate (*X*) using a sprayer equipped with flat spray tips (TeeJet 8001 VB), at 1.54 km h^{-1} and calibrated to deliver 127 L ha^{-1}. Plant response was evaluated 30 DAT, as described for the glyphosate resistance test. Survival was expressed as the percentage in relation to the control without application. The experiment was arranged as a completely randomized design, with four replications. Data were arcsin transformed (Eq. 1). ANOVA analysis and mean comparison Tukey's test were conducted with R3.0.2 statistical software.

Results

Habitat

The *B. napus* GER-resistant accession was found in three separate fields, owned by the same farmer. At the first surveyed point (site 1), the population formed a group of more than 1000 plants in a fallow field (Fig. 1). The group was arranged in a semicircular patch (>50 m^2) located less than 30 m from the *N* edge of a quadrangular field (~70 ha). Plants were at different phenological stages, and the average density was 5.4 ± 1.5 pl m^{-2} for mature plants, while the second cohort was at a density of 92.3 ± 10.3 pl m^{-2}. The field had received an application of glyphosate at the commercial dose rate (Table 2).

At the second surveyed point (site 2), the plants of the GER accession formed a patch of 50 × 20 m. This site was separated by less than 1000 m in a NO direction from site 1. The third site where a GER population was found (site 3) corresponded to a field separated by more than 12 km from sites 1 and 2. The population at this site formed a patch of more than 300 individuals at the flowering stage, at a density of 2.5 ± 1.6 pl m^{-2}, in a depression in a wheat field (~80 ha). Some isolated plants were observed on the road verges between the fields (site 4) that had survived a glyphosate application.

The main crop rotation in the fields was wheat soybean. The production system was under no tillage and chemical fallow with glyphosate and 2,4-D. Weed control in the wheat was done with metsulfuron-metil, 2,4-D and the mixture iodosulfuron + metsulfuron-metil (Hussar®). RR soybean was treated with glyphosate (Roundup Full II 66.2 %) at doses between 1.5 and 2 L ha^{-1}.

According to farmers' records, the resistant population had been present in the region for more than 3 years. It was first observed at site 3, and the dispersion was attributed to hired harvesting machinery. There were no records of oilseed rape crops in any of the fields over recent years. No other Brassicaceae crop were observed in adjacent fields or private gardens. Near site 2, some (<20) isolated *B. rapa* (wild turnip) plants were found inside a barley field, at the beginning of flowering.

The first field where the SMA population was found (site 5) was planted with RR soybean. The distance from site 1 was about 33 km. Plants were at multiple development stages. In a second soybean field (site 6), *B. napus* plants were flowering in the crop. The field received an application of glyphosate + imazethapyr (3 L ha^{-1} + 1.35 L ha^{-1}) and the phytotoxicity of imazethapyr was visible. These two fields belonged to the same farmer and the normal crop rotation was soybean wheat. Isolated plants were observed on the edges of roads, windmills, and other non-arable areas. In the corner of a field, next to a windmill and tank (site 7), a group of plants were found at the ripening stage. This site was separated by 25 km from site 5 and 10 km from site 1, representing an intermediate position between the two sites.

In a neighboring field (site 8), a patch of *B. napus* plants was observed in the corner of a RR soybean crop. The accession was identified as REG. According to information provided by the farmer, the first observations of these populations occurred in 2006–2007 on a field that was cultivated with

Table 1 Herbicides used to evaluate alternative control of glyphosate-resistant oilseed rape (*Brassica napus*) populations

Chemical family	Active ingredient	Trade name	Loading	Field rate (g AI ha^{-1})
Imidazolinone	Imazethapyr	Pivot	100 g L^{-1}	100.0
	Imazapyr	Arsenal	480 g L^{-1}	80.2
Sulfonylurea	Chlorimuron	Backup	250 g kg^{-1}	15.0
Triazolpyrimidine	Diclosulam	Spider	840 g kg^{-1}	25.2
Phosphinic acid	Glufosinate	Liberty	200 g L^{-1}	400.0
Benzothiadiazole nitrile	Bentazon	Basagran 60	600 g L^{-1}	960.0
Phenoxy	2,4-D	Genérico	602 g L^{-1}	451.5
Carboxylic acid	Fluroxypyr	Starane	288 g L^{-1}	172.8
Phenyltrifluoromethyluracil + imidazolinone	Saflufenacil + imazetapir	Optill	178 g kg^{-1} + 502 g kg^{-1}	24.9 + 70.3

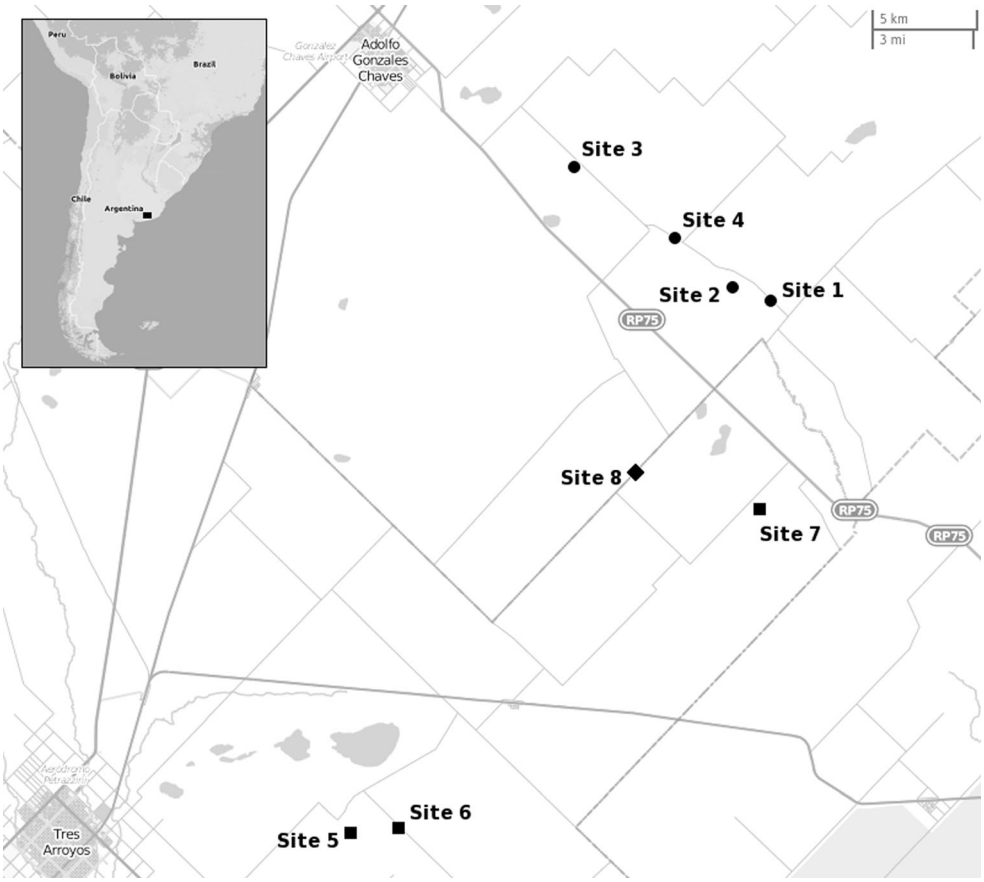

Fig. 1 Sites where feral oilseed rape (*Brassica napus*) accessions GER (*filled circles*), SMA (*filled squares*), and REG (*filled diamonds*) were found

oilseed rape of unknown origin. Emerging volunteers were highly resistant to glyphosate and were dispersed to other fields by harvesting machinery.

Morphological and chemical characterization

The morphological characterization showed that all accessions corresponded to the species *B. napus*. The most relevant qualitative characteristics for differentiating the species are leaf color, pubescence, clamping of upper leaves, flower color, disposition of buds in respect to open flowers, and the arrangement of mature siliques, and they were consistent with *B. napus* and different from the wild species *B. rapa*.

Principal component analysis of quantitative traits showed that the GER-resistant accession was morphologically similar to *B. napus* cultivars and different from all *B. rapa* wild populations (Fig. 2a). The beak/valve ratio was between 0.25 and 0.14 (0.21), the range accepted for *B. napus* species (Mulligan

Table 2 Oilseed rape (*Brassica napus*) accessions evaluated for glyphosate resistance

Accession	Site	Nearby town	Habitat	Population size	First year of detection	Oilseed rape cultivation	Crop rotation, herbicide use
GER	1, 2	Adolfo Gonzales Chaves	Within fallow fields. La Germana Co.	>1000 plants; 92.3 ± 10.3 pl m^{-2} (rosette); 5.4 ± 1.5 pl m^{-2} (flowering stage)	2009	Never	Wheat/soybean, wheat glyphosate, metsulfuron-metil, iodosulfuron, 2,4-D
	3		Within a wheat field. El Coraje Co.	2.5 ± 1.6 pl m^{-2}			
	4		On the edge of country road, which had received glyphosate applications.	1–5 plants			
SMA	5, 6	San Mayol	Within a RR soybean field.	>500 plants	2006	>5 years	Wheat/soybean, wheat glyphosate, metsulfuron-metil, imazethapyr
	7		Beside a mill, in a field corner.	~50 plants			
REG	8		Within a RR soybean field.	>500 plants			

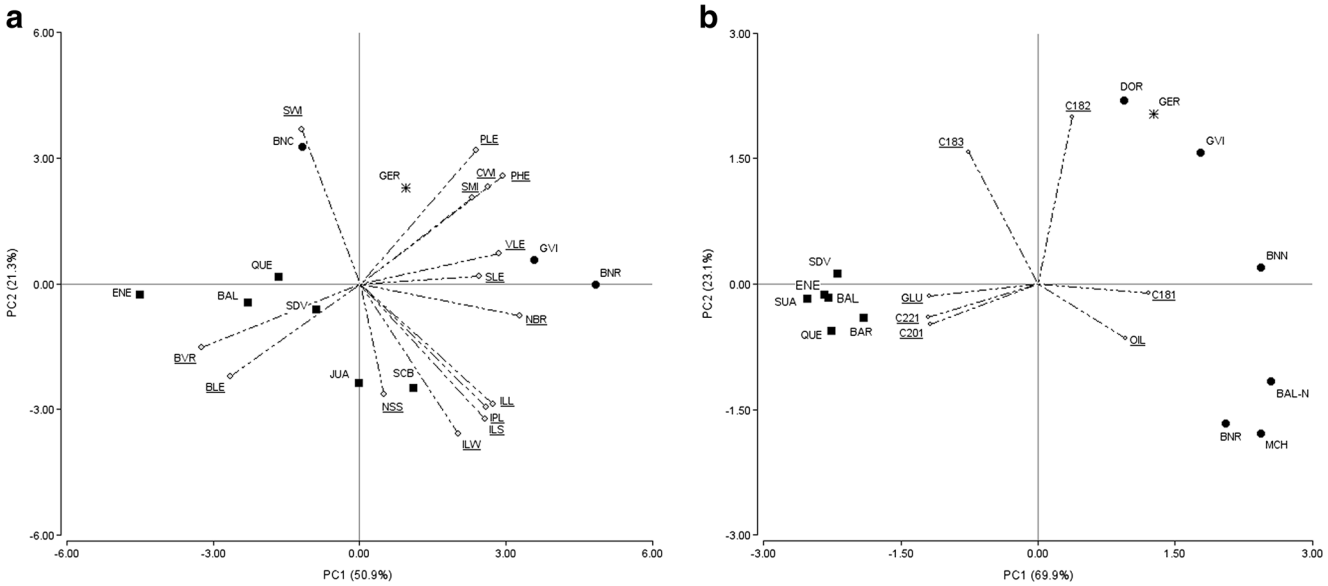

Fig. 2 Differentiation between oilseed rape (*Brassica napus*) susceptible (*filled circles*) and glyphosate-resistant (*asterisk*) accessions and wild *B. rapa* populations (*filled squares*) using principal component analysis for phenotypic characters (**a**) and oil composition traits (**b**). Parameters: **a** *CWI* cotyledon width, *PHE* mature plant height, *NBR* number of branches, *ILW* leaf width, *ILL* leaf length, *ILS* leaf size, *IPL* petiole length, *PLE* petal length, *SMI* number of siliques, *SLE* silique length, *SWI* silique width, *VLE* valve length, *BLE* beak length, *BVR* beak/valve ratio, *NSS* number of seeds per silique; **b** *OIL* oil concentration, *C181* oleic acid, *C182* linoleic acid, *C183* linolenic acid, *C201* eicosenoic acid, *C221* erucic acid, *GLU* glucosinolates

1995). A PCA with only *B. napus* accessions could not differentiate the GER population from the two oilseed rape cultivars and a volunteer population (Online resource 1, Fig. S1).

The oil composition was also similar in the *B. napus* cultivars and GER-resistant accession, although some differences between cultivars were also detected. This was the result of high levels of oleic acid in some canola cultivars. All of them were clearly different from the *B. rapa* wild populations that presented high levels of erucic acid and glucosinolates, components that were almost absent in canola cultivars and in the resistant accession (Fig. 2b).

Herbicide resistance characterization

All *B. napus* accessions (GER, SMA, and REG) were resistant to a double dose of glyphosate. Survival and dry matter was near or above 90 %, differing from the conventional oilseed rape cultivar which showed severe symptoms of phytotoxicity. No significant differences between resistant accessions were found (Table 3).

Dose-response assay confirmed that the GER accession was highly glyphosate resistance (Fig. 3a). Survival was greater than 70 % even at 15-fold the commercial dose rate of glyphosate. LD_{50} of GER was 27.40 kg a.e. ha^{-1} (21*X*), while BNC was 0.48 kg a.e. ha^{-1}. This represented a resistance factor of 57 (Table 4). These results were similar to those obtained from the dry matter (Fig. 3b). GR_{50} for GER accession was estimated at 14.76 kg a.e. ha^{-1} whereas for conventional oilseed rape cultivar BNC was 0.05 kg a.e. ha^{-1}, with a resistance factor of 311 (Table 4).

Transgene detection

All plants of the SMA and GER accessions sampled showed positive response to the immunological test, confirming that they possessed the CP4 EPSPS enzyme from *A. tumefaciens*. All individuals of the conventional oilseed rape cultivar (BNC) were negative to the test (Online resource 1, Fig. S2).

All sampled individuals of the GER accession amplified the 108-bp fragment corresponding to the GT73 event, consistent with the occurrence of glyphosate-resistant *B. napus* accessions. However, the five individuals of the conventional cultivar BNC did not amplify this fragment (Online resource 1, Fig. S3).

Table 3 Plant survival and dry matter, expressed as the percentage of the untreated control (mean ± standard error) of oilseed rape (*Brassica napus*) populations GER, SMA, and REG and a conventional canola cultivar (BNC), after the application of glyphosate at double the commercial rate (2*X*)

Accession	Survival (%)	Dry matter (%)
BNC	0.0 ± 0.0 a	0.0 ± 0.0 a
GER (site 1)	100.0 ± 0.0 b	88.0 ± 5.8 b
SMA (site 7)	96.9 ± 2.4 b	89.8 ± 4.2 b
REG (site 8)	86.3 ± 6.3 b	103.0 ± 11.7 b

In each column, different letters indicate significant differences according to Tukey's test ($P < 0.05$)

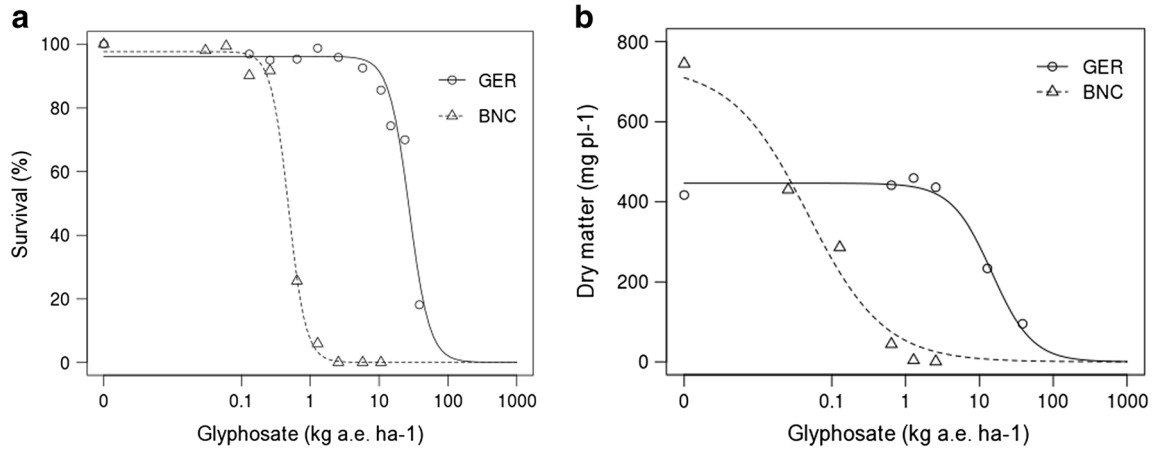

Fig. 3 Response of the susceptible (*empty triangles*) and the resistant (*empty circles*) oilseed rape (*Brassica napus*) accessions to the application of glyphosate, expressed as percentage survival (**a**) and milligrams of dry matter per plant (**b**)

Alternative herbicides

All evaluated herbicides showed complete control of the GER-resistant accession, at a commercial dose rate. AHAS-inhibiting herbicides (imazethapyr, imazapyr, chlorimuron, and diclosulam) were slower in showing phytotoxicity symptoms and some dry matter was left. Glufosinate provided an excellent control of all plants. Hormonal herbicides (2,4-D and fluroxypyr) were highly effective and fast-acting. A mixture of saflufenacil and imazethapyr mixture was equally effective. No significant differences were found in plant survival between the different treatments and biotypes.

Discussion

The uncontrolled *B. napus* populations showed high resistance to glyphosate, even at 30-fold the commercial dose. The resistance profile of the GER accession measured in the greenhouse was equivalent to that observed by (Nandula et al. 2007) in a transgenic glyphosate-resistant canola cultivar (Hyola 514RR). The species was identified as *B. napus*, with traits of canola cultivars (like low erucic acid and glucosinolates content), on account of the morphological and chemical characterization of the GER plants.

The immunological and molecular analyses showed that these accessions presented the GT73 transgenic event that confers glyphosate resistance. The origin of this event in the accessions is uncertain, as the cultivation of transgenic oilseed rape cultivars is prohibited in Argentina. This might suggest that glyphosate resistance could come from unauthorized transgenic oilseed rape crops cultivated in the country. Some farmers and technicians who were consulted revealed that the problem appeared in rented land with a history of oilseed rape production whose origin cannot be tracked, or even after the cultivation of conventional canola cultivars in other regions (Repetto, personal communication).

The transgenic plants could also be introduced into the country as seed contaminants in oilseed rape cultivars imported before 2007, the year in which OGM analysis was made mandatory (Secretaría Nacional de Sanidad Ambiental (SENASA) 2007). All oilseed rape cultivars commercialized in Argentina are introductions made from the principal breeding centers of the world, and multiplied in the country, as there is no national breeding program for the crop. An important number of oilseed rape cultivars sown in Argentina come from countries with extensive production of RR oilseed rape, like Canada, the USA, and Australia (Iriarte 2015; Instituto Nacional de Semillas (INASE) 2016). In October 2005, tolerance levels for GM canola in non-GM canola in Australia and other Organization for Economic Co-operation and Development (OECD) member countries were set at 0.9 % for grain and 0.5 % for seed for sowing (OECD 2007). Friesen et al. (2003) found conventional canola seedlots in Canada with GM contamination levels above 2.0 %. In Switzerland,

Table 4 Estimated parameters for non-linear regression equations comparing survival and dry matter from resistant (GER) and susceptible (BNC) oilseed rape (*Brassica napus*) accessions to glyphosate. Comparative levels of resistance were also estimated

Accession	*b*	*d*	*e* (LD_{50} or GR_{50})	RF
Survival (%)				
BNC	3.46	97.65	0.48	56.87
GER	2.95	96.14	27.40	
Dry matter (mg pl^{-1})				
BNC	0.84	737.53	0.05	310.94
GER	1.58	446.29	14.76	

Parameters: *b* slope of the curves around *e*, *d* upper limit of curves, *e* inflection point of the curves, LD_{50} and GR_{50} effective rate (kg a.e. ha^{-1}) required for 50 % reduction in plant survival and dry matter, respectively, *RF* resistance factor (LD_{50} or GR_{50} R/LD_{50} or GR_{50} S)

the presence of GM oilseed rape plants around port areas was attributed to seed contaminants in imported Canadian wheat (Schulze et al. 2015). Imports of cereals and oilseed for production in Argentina are negligible (FAOSTAT 2016).

The presence of transgenic *B. napus* populations invading farms with no history of oilseed rape cultivation could be caused by seed movement. The farmers and technicians consulted thought that the emergence and spread of these resistant populations was due to the dispersion of seed by machinery. This cannot be ruled out since it is known that the agricultural machines can participate in weed dispersion (Benvenuti 2007; Michael et al. 2010). In Argentina, approximately 47 % of harvest is undertaken with hired machinery (Piñeiro and Villarreal 2005). This is a common occurrence in this species because of the characteristics of *B. napus* seeds (von der Lippe and Kowarik 2007; Bailleul et al. 2012; Allnutt et al. 2013).

Feral *B. napus* populations found elsewhere in the world appear frequently in ruderal habitats and near to places with intensive oilseed rape cultivation (Devos et al. 2012). In some cases, it has been proven that these populations persist for several years, involving processes of population movements, density changes, extinction and recolonization (Crawley and Brown 2004). Pivard et al. (2008b) showed that the persistence of feral populations could be equally due to the constant supply of seeds from cultivated fields nearby and the persistence of the seed bank in the soil. However, they also found that a smaller percentage could be attributed to local recruitment which contributed to the establishment of self-perpetuating populations.

The case presented in this study is of particular characteristics. While the presence of transgenic *B. napus* feral plants has been reported in countries where the cultivation of GM oilseed rape was forbidden (Saji et al. 2005; Schoenenberger and D'Andrea 2012; Hecht et al. 2014), these plants appeared in seed handling areas such as ports, roads, and railways. In our country the transgenic plants were detected in typically ruderal habitats, acting as weeds and being dispersed for several kilometers without the mediation of oilseed crops.

Experimentation showed that there are alternative herbicides for controlling resistant *B. napus* populations in various situations and crops. No resistance to glufosinate or AHAS-inhibiting herbicides, traits present in other GM HR canola cultivars, was found. In RR soybean cultivars, AHAS-inhibiting herbicides (imazethapyr, chlorimuron, and diclosulam) may be recommended. In winter cereals, 2,4-D proved to be very effective and could be used for the early control of the first emergence of the population. In more advanced stages of cultivation, fluroxypyr would be recommended to control subsequent emergency that had escaped the early control.

Herbicides evaluated in this research were employed by farmers in one of the fields invaded with this biotype and were effective in controlling the weed. Monitoring of this field showed no evidence of the population by fall 2014 (site 1). Absence of plants was confirmed again in 2016. In contrast, no management modification was performed at site 3 and glyphosate was used as a primary control method. At this place, the resistant *B. napus* population had spread throughout the field where it had been located in 2012 and also to a neighboring field. The population was estimated at over 100,000 plants by 2014. In the successive years, tillage was practiced in this field and by 2016 no plants of resistant oilseed rape were found.

The presence of transgenic *B. napus* populations with glyphosate resistance in Argentina reaffirms the importance of herbicide rotation. The invasiveness of these biotypes in agricultural environments is enhanced by the presence of herbicide resistance. This character gives it a distinct advantage in farming systems based on intensive use of glyphosate. Furthermore, the presence of these populations raises concern about the potential for hybridization with wild related species, especially with *B. rapa.* This species is widely distributed in the region where GM HR *B. napus* was found. This risk was the main argument used by the national authorities to ban the cultivation and import of GM canola cultivars.

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