Against the current - Clearfield® oilseed rape in Germany

Gegen den Strom – Clearfield-Raps in Deutschland

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Abstract

The introduction of imidazolinone-tolerant oilseed rape (Clearfield® system, CL; OSR) meets with scepticism in Germany. Frequently mentioned as a major drawback for growing CL-OSR in Germany is the possible occurrence of herbicide resistant CL-volunteers which could run out of control. A monitoring was started by BASF in the seasons 2011/2012/2013 on farms in Germany prior to commercial launching of the Clearfield® system. Aim of this phase was, among others, to analyse the potential for occurrence and survival of volunteers, and to identify factors which promote or minimize volunteers. The University of Hohenheim monitored 41 farms in nearly all regions of Germany during spring 2013 in the crops following to CL-OSR (harvested in summer 2012), mostly winter wheat. Volunteer OSR was counted on the fields and molecular-biologically analysed, and the soil seed bank was investigated in 0 – 30 cm soil depth. Additionally, data about field history and cultivation were gathered. Several CL-varieties were tested at the same time in the laboratory for secondary dormancy. First results showed variation in dormancy from high (>90%) to nearly no dormancy of the tested CL-varieties, similar to common varieties. The situation on farmers fields corresponded to dormancy values: 40 – 49% of all farms had no CL-soil seedbank or volunteers at all, and a maximum of 1000 CL seeds m^{-2} and two CL-volunteers m^{-2} were observed on the other farms. Conventional seed banks and volunteers additionally occurred on the majority of the farms. Overall, seed survival and volunteer emergence of CL-OSR can be assessed as medium-low compared to previous studies with conventional varieties. Volunteers observed after CL growing do not necessarily originate from this crop. The regression analysis showed no clear effects, probably because of the low number of sampling sites. This monitoring did not reveal specific disadvantages linked with CL-oilseed rape in terms of volunteers.

Keywords: *Brassica napus*, Clearfield®, dormancy, soil seed bank, variety, volunteers

Zusammenfassung

Die Einführung von Imidazolinon-tolerantem Raps (Clearfield®-System, CL-Raps) stößt in Deutschland auf Skepsis. Ein häufig genannter Nachteil des CL-Rapses in Deutschland ist das mögliche Auftreten von herbizid-tolerantem und schwer bekämpfbarem CL-Durchwuchs. Daher wurde von der BASF in den Jahren 2011/2012/2013 vor der Einführung des Clearfield®-Systems ein Probeanbau und ein Monitoring auf landwirtschaftlichen Betrieben in ganz Deutschland vorgenommen. Ziel war unter anderem, das Potential für das Auftreten von Durchwuchsraps zu analysieren und Faktoren zu identifizieren, die zu Durchwuchsraps führen. Die Universität Hohenheim hat bei diesem Monitoring im Frühjahr 2013 Flächen (überwiegend Winterweizen) auf 41 Betrieben in ganz Deutschland untersucht, auf denen im Vorjahr CL-Raps geerntet worden war. Durchwuchsraps wurde gezählt und molekularbiologisch analysiert; weiterhin erfolgte eine Bodenprobennahme in einer Bodentiefe von 0 – 30 cm auf 20 Flächen zur Bestimmung des Bodensamenvorrats an Raps. Über einen Fragebogen wurden zusätzliche Informationen zu Schlaghistorie und Anbauverfahren abgefragt. Parallel dazu wurden die angebauten sowie weitere CL-Sorten im Labor auf sekundäre Dormanz getestet. Zwischen den Sorten zeigten sich die schon bei konventionellem Raps bekannten Unterschiede in der Dormanz, die von hoch dormant (> 90 %) bis nahezu nicht dormant reichten. Auf den Praxisbetrieben ergab sich ein ähnliches Bild: auf 40 – 49 % der Betriebe wurden weder ein CL-Bodensamenvorrat bzw. noch CL-Durchwuchsraps gefunden. Der Durchwuchs auf den anderen Betrieben belief sich auf maximal rund zwei CL-Durchwuchspflanzen m^{-2}, und knapp 1000 CL-Samen m^{-2} waren maximal im Samenvorrat. Auf den meisten Flächen fanden sich zusätzlich konventioneller Durchwuchsraps bzw. Bodensamenvorrat oder Kreuzungen zwischen konventionellem und CL-Raps. Insgesamt kann der Besatz mit CL-Durchwuchs im Vergleich zu Studien mit konventionellem Durchwuchs als mittel bis gering eingestuft werden. Die Regressionsanalyse ergab bei der geringen Anzahl geprüfter Flächen wenig einheitliche und eindeutige Ergebnisse. Mit dem Anbau von CL-Raps scheinen auf den Monitoringflächen keine besonderen Nachteile im Hinblick auf Durchwuchsraps verbunden gewesen zu sein.

Stichwörter: *Brassica napus*, Bodensamenvorrat, Clearfield®, Dormanz, Durchwuchs, Sorte

Introduction

Clearfield® (CL) is worldwide the common term for the combination of an imidazolinone-based herbicide and a corresponding plant which is tolerant against the active ingredient of the herbicide. The aim of the CL-system in oilseed rape (OSR) was to reliably control broadleaf- and grass weeds in OSR in post-emergence. With this system it is possible to control especially crucifers in OSR, for example bittercress (*Erysimum barbarea* L.), turkish rocket (*Bunias orientalis* L.) or white top (*Lepidium draba* L.) which was not possible in OSR with other herbicides before. Also the control of volunteer OSR from traditional genotypes in the crop rotation is possible with the CL-system (CHENEVIER *et al.*, 2013). In March 2012 the herbicide Clearfield®-Vantiga® D (imazamox) was officially approved in Germany (CHENEVIER *et al.*, 2013), which enabled the introduction of the CL-OSR system in Germany. CL-OSR has a tolerance against the active ingredient imazamox which belongs to the mode of action of branched chain amino acid synthesis (ALS) inhibitors.

Due to the ability of rapeseed seeds to stay dormant in the soil for up to 11 years (LUTMAN *et al.*, 2003), rural administration, regional authorities and official plant protection services raise concern about the system (NIEHOFF and KLINGENHAGEN, 2012). The scepticism about this system is – partly – caused by the possible occurrence of imazamox-tolerant volunteers in the cropping system. One of the debated reasons for volunteers is the outcrossing of CL-OSR with conventional OSR or even with other species of the same family. Due to that it could be possible that the tolerance can spread into other OSR fields (HÜSKEN and DIETZ-PFEILSTETTER, 2007) or in the environment in general (Liu *et al.*, 2013) The same can happen if farmers share machinery and the combine harvester unintendedly transports remaining CL-seeds to another field (NIEHOFF and KLINGENHAGEN, 2012). Most relevant, however, is the fact that CL-volunteers from the high number of seeds lost during harvesting can emerge in following crops, including in OSR, which are most probably tolerant against several herbicides with similar mode of action as the herbicide Vantiga®. BASF has started a testing phase of growing CL-OSR with selected farms in Germany prior to the commercial introduction of the herbicide and the varieties. Among agronomical traits of the varieties and efficiency of the herbicide, experience should be gathered about seed survival in the soil and the amount of CL-volunteers in the crop rotation. The University of Hohenheim has experience in the research about volunteer oilseed rape for more than 15 years (Gruber *et al.*, 2004; PEKRUN *et al.*, 2005; WEBER *et al.*, 2013). It is well known that the level of seed dormancy (PEKRUN *et al.*, 1997; GRUBER, 2004) determines the size of the soil seed bank and the number of volunteers which emerge from this soil seed bank. Furthermore, experiments have shown that post-harvest tillage is an important issue in this context: incorporation of seeds from harvest loss quite soon after harvesting of oilseed rape often induces secondary dormancy, promotes the creation of a longstanding soil seed bank and thus promotes the occurrence of volunteers (GRUBER *et al.*, 2010). Other factors such as climate or soil type are not yet well studied. The evaluation of the soil seed bank and volunteers from many practical farms all over Germany offers a good opportunity to crosscheck the results from field trials in agricultural practice, and to gain information about other factors which have an impact on seed survival of oilseed rape in the soil. Aim of the study was to analyse and to describe the soil seed bank and number of volunteers after the cultivation of CL-oilseed rape, and to identify factors which have facilitated seed survival and volunteers.

Material and Methods

The monitoring was conducted from April 29th till July 5th 2013 from southern Germany to northern Germany. The sampling sites were distributed throughout all Federal States of Germany except Berlin, Bremen, Hamburg and Saarland, with a focus on the areas typical for OSR cultivation (Fig. 1). Additionally, a questionnaire was handed to the farmers to gain further information about the history and cultivation practice of the fields.

Sampling Sites

As a whole, 41 sites (farms) were evaluated on which CL-OSR was cultivated in the growing period 2011/2012. The CL-OSR growing area varied from 0.68 to 8.0 hectares (ha) with an area of 3.25 ha on average. Only one variety was grown on each experimental site. All together, six different CL-varieties with different potential for secondary dormancy (tested according to WEBER *et al.*, 2010) were grown. The sowing and cultivation was done according to best management practice and according to the different local conditions. Thus the sampling sites were distributed all over Germany, and a wide range of different soil types, climate conditions and management practices were covered. The following crop was mostly winter wheat (32 sites), but also winter barley (six sites), winter triticale (one site) and winter rye (two sites) were grown.

Fig. 1 Locations of the sampling sites in Germany; STEPMAP (2013).

Abb. 1 *Lage der Untersuchungsflächen in Deutschland; STEPMAP (2013).*

Soil Samples

To determine the soil seed bank, soil samples were taken from 20 out of the 41 sites. The soil samples were taken with a PUERCKHAUER auger having a diameter of 1.6 cm. On each sampling site 40 cores from 0-30 cm were randomly taken from the whole CL-OSR growing area. The samples were collected as bulk samples of three different depths (0–10 cm, 10–20 cm and 20–30 cm). To suppress seed germination, the soil samples were immediately cooled down and transferred into a deep freezer as soon as possible. The soil samples were washed out using a FRITSCH (Fritsch GmbH, Germany) analytic sieve with a mesh width of 1 mm. Afterwards the OSR seeds were hand-selected from the sieve residues. The seeds were sent to a laboratory for DNA analyses.

Evaluation for Volunteers

All 41 sites were examined for volunteer OSR. On each site a frame with an area of one square meter was randomly placed 20 times. In each frame the OSR volunteers were counted and each individual plant was collected. To identify CL-OSR volunteers among all volunteers, 2–4 discs with 6 mm diameter were cut out from the youngest leaflets with a Harris UNI-CORE™. To avoid cross-contamination between the different plant samples, the Harris UNI-CORE™ was cleaned using 90% ethanol after each individual plant. The discs of each plant were dried and afterwards inserted into a 96-well-plate and sent by express to a laboratory for DNA analyses.

Molecular –Biological Analysis

The DNA analyses were done by the external laboratory ScanBi Diagnostics (ScanBi Diagnostics, Arnalb, Sweden). The genomic DNA (gDNA) was extracted from the leaf and seed material and eluted in water. Afterwards the gDNA was pre-amplified by polymerase chain reaction (PCR) using gene specific oligonucleotide primers targeting either the BnAHASL1A gene which is the target of PM2 resistance or the BnAHASL1C gene which is the target of PM1 resistance and a polymerase with proofreading activity. The resulting PCR product was diluted with water. The samples were subsequently processed in a high throughput manner using automation to identify the zygosity at the nucleotide sites responsible for imidazolinone resistance (RUTLEDGE *et al.*, 1991). The assay, employing real-time PCR technology for single nucleotide polymorphism (SNP) identification (HOLLAND *et al.*, 1991), utilizes minor groove binder (MGB) type probes. For each assay to detect either the PM1 or PM2 genes, two probes were designed to differentiate the two alleles and each probe was bound to a different fluorescent molecule independently detected within a duplex PCR. One probe was designed to bind specifically to the resistant or mutant allele and the other one specifically targets the susceptible or non-mutant allele. The two probes generate fluorescent signals with different wavelengths which were captured in real time by an ABI 7900HT thermal cycler. The measurement for each assay was then compared to a standard control of known genotype to identify if any mutation either in PM1 or PM2 or both can be proven or not.

Statistical Analysis

Based on the small dataset it was necessary to confine the evaluation to those effects which are known to have a high influence on the soil seed bank and volunteers of OSR. As a first step the most important effects, with possible impact on both volunteer CL-OSR and the soil seed bank, were pre-selected from the questionnaires. The cultivar was set per definition as fixed effect. Additionally, the following effects were included in the multiple regression analyses: soil type, time of the first post-harvest tillage, depth of the first, second and third post-harvest tillage, pre- and post-harvest seed losses, yield of the CL-OSR, and the factor inversion tillage or non-inversion tillage after CL-OSR. The multiple regression analyses were performed using the procedure PROC REG with stepwise selection of the statistical software SAS version 9.3 (SAS Institute Inc., Cary/North Carolina) to use a selected model for further tests after that.

To test whether the pre-selected effects have different influence on CL-OSR and crossbreed CL-OSR the dataset was subdivided into these two parts. The multiple regression using stepwise selection procedure was progressed separately for each part. For the crossbreed CL-OSR additional variable effects such as years after last OSR cultivation, frequency of soil inversion tillage after the last OSR, the possibility of occurrence of OSR in CL-OSR and OSR on the adjacent area were added.

To perform F-tests for the fixed effects and multiple t-tests for the fixed and selected ($\alpha = 0.15$) effects of the multiple regression, the selected model was analyzed with a mixed model (PROC MIXED) with $\alpha = 0.05$.
The following model was used: $y = X\beta + e$
γ = vector of seeds m^{-2} or volunteers m^{-2} of each part
β = vector of fixed effects and covariables
X= design matrix
e = vector of the residual error

Results

OSR soil seed bank

CL-OSR seeds were found on eight of the 20 sampling sites (40% of all sites, Tab. 1). The highest amount was found on a field in Saxony (SN) with 995 seeds m^{-2}. Except of the sites in Bavaria (BY), Rhineland-Palatinate (RP), North Rhine-Westphalia (NRW), Lower Saxony (NI) and Mecklenburg-Western Pomerania (MV) where no CL-OSR seeds were found, the smallest soil seed banks with 124 CL-OSR seeds m^{-2} were in Baden-Wuerttemberg (BW), Saxony-Anhalt (ST) and Brandenburg (BB). On average 162 CL-OSR m^{-2} could be found on the examined sites in Germany.

Tab. 1 Number of CL- and crossbreed OSR seeds m^{-2} in the soil seed bank on farmers' fields in federal states of Germany, in first spring (year 2013) after harvesting CL-OSR.

Tab. 1 *Anzahl an CL- und ausgekreuzten Rapssamen m^{-2} im Bodensamenvorrat auf Praxisflächen im ersten Frühjahr (2013) nach dem Anbau von CL Raps, angegeben nach Bundesländern.*

Federal states	**BW**	**BY**	**RP**	**TH**	**ST**	**SN**	**BB**	**NRW**	**NI**	**MV**	**SH**
No. sampling sites	2	3	1	3	3	1	1	1	1	1	3
CL seeds m-^{2} on each site	124	0	0	249	124	995	124	0	0	0	249
	0	0		373	497						0
		0		0	0						0
Crossbreed seeds m-^{2} on each site	0	124	0	0	0	0	249	0	0	0	124
	0	124		0	0						0
		0		0	0						0

Besides pure CL seeds, also crossbreed seeds from outcrossing of CL and non-CL OSR were found in BY, BB and SH (Schleswig-Holstein), with a maximum of 249 seeds m^{-2} (Tab. 1). Conventional OSR (not shown) was found in total on 11 sites in every federal state except in NRW, NI and MV. The highest number of conventional OSR in the soil seed bank was found in SH with 1243 seeds m^{-2}, a value which exceeded the number of CL-seeds. These seeds must have derived from conventional OSR cultivation at least two, or even more, years ago.

The levels of secondary dormancy of the CL-OSR varieties grown by the farmers, determined by the Hohenheim Standard Dormancy Test in the laboratory (data not shown), and the mean number of CL-OSR seeds m^{-2} correlated positively and quite closely with $R^2 = 0.67$.

Volunteer OSR

Though many of the farmers had used ALS inhibitors (which would not strongly damage the CL volunteers) for weed control in winter wheat, many farms had no volunteers at all. At 20 out of 41 monitored sites, no volunteer CL-OSR was observed (Tab. 2), which is corresponding to 49% of all the sites. Most volunteers were found on a site in the federal state of Thuringia (TH) with a mean of

2.05 CL-OSR volunteers m^{-2}. The majority of sites where volunteers were observed had less than 0.5 CL-OSR volunteers m^{-2}. Averaged over all sites there were 0.27 CL-OSR volunteers m^{-2}. Crossbreed CL-OSR also occurred at some sites, obviously from pollen flow from conventional oilseed rape outside the CL-field to the CL-OSR. With exception of the sites in BW and ST with 0.45 and 0.25 crossbreed volunteers m^{-2}, there were usually < 0.1 crossbreed volunteers m^{-2} found. More than 50% of the sites had no conventional volunteers (data not shown), and the highest number was found in winter barley on a site in BB with 1.15 plants m^{-2}. In average 68% of all volunteers found were CL-OSR from directly preceding harvest, and 25% were conventional OSR volunteers probably from years before. Dormancy levels of the varieties and the mean of CL-OSR volunteers m^{-2} correlated positively with $R^2 = 0.73$.

Tab. 2 Number of CL- and crossbreed OSR volunteers m^{-2} found on farmers' fields in the federal states of Germany, in first spring (2013) after harvesting CL-OSR.

Tab. 2 *Anzahl an CL- und ausgekreuztem Durchwuchsraps m^{-2} auf Praxisflächen im ersten Frühjahr (2013) nach dem Anbau von CL-Raps, angegeben nach Bundesländern.*

Federal state	**BW**	**BY**	**RP**	**HE**	**TH**	**ST**	**SN**	**BB**	**NRW**	**NI**	**MV**	**SH**
No. of sampling sites	2	5	2	2	6	5	4	2	1	2	6	4
	0.60	0.70	0.20	0.00	2.05	1.90	0. 05	0.45	1.85	0.00	0.10	0.15
	0.00	0.55	0.00	0.00	0.15	1.60	0.05	0.15		0.00	0.00	0.00
CL- volunteers m-^{2} on each site		0.15			0.15	0.00	0.05				0.00	0.00
		0.10			0.05	0.00	0.00				0.00	0.00
		0.00			0.05	0.00					0.00	
					0.00						0.00	
	0.45	0.05	0.00	0.00	0.05	0.25	0.05	0.10	0.05	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00		0.00	0.00	0.00
Crossbreed volunteers m-^{2} on each site		0.00			0.00	0.05	0.00				0.00	0.00
		0.00			0.00	0.00	0.00				0.00	0.00
		0.00			0.00	0.00					0.00	
					0.00						0.00	

<u>Factors affecting soil seed bank and volunteers</u>

The statistical analyses revealed no clear and consistent results on factors affecting the soil seedbank and the number of volunteers, mainly because of the high variability of the data set which is due to the small number of sampling sites (only 41). Delayed first post-harvest tillage resulted in lower numbers of volunteers and a lower soil seed bank than immediate tillage, though it was not statistically significant. The soil seed bank of crossbreed seeds was significantly highest in sandy soils. There were effects of the CL-variety on the CL-soil seedbank and the number of CL-volunteers as well, but also below significance level. High-dormancy varieties (grown only on few of the monitored farms) resulted in comparatively high numbers of volunteers. Primary tillage deeper 10 cm tended to result in a higher soil seed bank than shallow primary tillage, independently from the mode of tillage (inversion or non-inversion tillage).

Discussion

There was a high variability in the number of CL-OSR volunteers, and in the amount of CL-seeds in the soil seed bank as well. In contrast to our expectation nearly the half of all sampling sites was free from CL-OSR volunteers and free from CL-OSR seeds surviving in the soil, as far we could observe. The amount of volunteers and seed survival is in accordance with results from previous studies about conventional OSR (GRUBER *et al.,* 2005) who found the soil seed bank in the first year after OSR consisting of several hundred seeds, and volunteers' numbers of <1.3 plants m^{-2}.

The occurrence or absence of CL-OSR seeds and volunteers m^{-2} on the fields can be explained by the different disposition of OSR varieties to acquire secondary dormancy under specific environmental conditions. Seed dormancy is the most relevant factor for the existence of a soil seed bank and thus also for volunteers which emerge from the soil seed bank in the course of time (PEKRUN *et al.*, 1997). Similar results were shown by different researches (LUTMAN *et al.*, 2003; GRUBER *et al.*, 2004; GRUBER *et al.*, 2010; WEBER *et al.*, 2013). The quite high correlation between the disposition of the CL varieties to secondary dormancy and the size of the soil seed bank emphasizes the effect of dormancy. The variety with the highest dormancy level resulted in the greatest soil seed bank. The correlation was even higher between dormancy and volunteers compared to the correlation of dormancy-soil-seedbank, so that most of the variability in the occurrence of volunteers can in fact be explained by dormancy.

Other factors such as time and depth of tillage (GRUBER *et al.*, 2010; PEKRUN and HUBERT, 2012), or soil texture had most likely an additional effect on seed survival and volunteers' emergence. These effects, however, could not reliably be calculated due to the comparatively small number of sites, and the great number of variables, such as different agronomic measures and different weather conditions at the farms.

Although the CL-OSR was often cultivated on a sub-area within a conventional OSR field, an average of only 7% of all volunteers across all sampling sites were crossbreeds between conventional and CL-OSR. The outcrossing rate of herbicide-resistant OSR depends mainly on the variety and is also dependent on the local conditions and the topography (HÜSKEN and DIETZ-PFEILSTETTER, 2007).

An assumed, insufficient chemical control of CL-OSR volunteers is another point of criticism raised by the public. In 34% of the sample sites herbicides with ALS inhibitors as active ingredient or ALS inhibitors as parts of the herbicides were applied. Nevertheless most of these farms had no or very few CL-volunteers on their fields. This result gives evidence that the control of CL-OSR obviously does not seem that very problematic as often assumed (NIEHOFF and KLINGENHAGEN, 2012). All in all, the number of sites was too small to allow a final assessment of the issue of CL-volunteers. The study showed that CL-volunteers did not cause more weedy effects than conventional OSR did, in the given conditions of the sampling sites and of the year 2013. Farmers' practice, without knowing the details from this study which practice was most effective, is obviously often suitable to reduce conventional and CL-OSR volunteers nearly to zero. Variety-dependent dormancy appears to be worth to be considered for future breeding and growing CL oilseed rape. Low-dormancy varieties could well support other management measures to minimize volunteers.

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