Marker-assisted identification of oilseed rape volunteers in oilseed rape (*Brassica napus L.*) fields

Marker-vermittelte Identifizierung von Durchwuchsraps in Rapsfeldern

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Summary

Seed losses of oilseed rape (OSR) occur before and during harvest. Seeds can persist in soils for several years and often appear as volunteers in successive crops. Oilseed rape volunteers (OSRV) can harm the product quality if they emerge in subsequent OSR crops differing in fatty acid profile or other quality traits.

Several factors can affect volunteer abundance. Important factors identified under controlled conditions are OSR post-harvest management (stubble tillage) and OSR variety selection in terms of the genotypic secondary dormancy potential.

In the growing seasons 2009/10 and 2010/11, OSR volunteer abundance was surveyed on agricultural OSR fields in Germany. The main objective was to assess factors affecting volunteer abundance under on-farm conditions by a prediction model. Volunteer numbers were determined by two different approaches: 1. Cultivation of a semi-dwarf hybrid OSR variety, and 2. Survey of OSR volunteers between wide rows in OSR crops. Data analysis taking into account farmers´ questionnaires revealed that the factor "variety selection" was not feasible for the prediction model. As an alternative approach to assess the impact of variety selection on volunteer abundance, the genotypic origin of volunteers was investigated by DNA fingerprints using ISSR (Inter Simple Sequence Repeat)-PCR. Molecular marker analysis confirmed that OSR volunteers were to a large extent identified correctly. In four of seven selected fields, plants could be assigned to open pollinating varieties cultivated previously. In two of these fields, a high dormancy (HD) variety was found to account for a large proportion of the volunteers. In contrast, low dormancy varieties appeared only rarely as volunteers. Hybrid varieties could never be identified, due to genetic segregation in the F_2 generation. Taken together, the results indicate that HD varieties substantially contribute to high OSR volunteer abundance in agricultural fields, although more data are needed to confirm this.

Keywords: *Brassica napus,* ISSR-PCR, secondary dormancy, seed persistence, volunteers, winter oilseed rape

Zusammenfassung

Samenverluste entstehen bei Winterraps vor und während der Ernte. Die Samen können im Boden für Jahre überdauern und erscheinen oft als Durchwuchsraps in Folgekulturen. Durchwuchsraps gentechnisch veränderter Sorten kann die Produktqualität schmälern, wenn er in nachfolgend angebautem Raps mit verändertem Fettsäuremuster oder anderen speziellen Qualitätsmerkmalen auftritt.

Mehrere Faktoren können die Abundanz von Durchwuchsraps beeinflussen. Wichtige Einflussfaktoren, die unter kontrollierten Bedingungen festgestellt wurden, sind die Stoppelbearbeitung nach der Rapsernte und die Rapssortenwahl auf Grund der genotypisch beeinflussten sekundären Dormanz.

In den Anbaujahren 2009/10 und 2010/11 wurde auf Rapsfeldern in Deutschland das Auftreten von Durchwuchsraps untersucht. Hauptziel war es, Ergebnisse aus vorherigen Studien durch ein Prognosemodell für Durchwuchsraps unter Praxisbedingungen zu bestätigen. Die Abundanz von Durchwuchsraps wurde mit zwei Methoden ermittelt: 1. Aussaat einer Halbzwerg-Hybridrapssorte, und 2. Erhebung von Durchwuchsraps in Rapsfeldern mit großen Saatreihenabständen. In der statistischen Auswertung unter Einbeziehung der von den Landwirten erhaltenen Informationen erwies sich der Faktor „Rapssortenwahl" jedoch auf Grund der Vielzahl der nacheinander angebauten Sorten als unbrauchbar. Alternativ wurde daher die Sortenherkunft von Durchwuchsrapspflanzen mit Hilfe von genetischen Fingerabdrücken unter Verwendung der ISSR (Inter Simple Sequence Repeat)-PCR untersucht, um auf diese Weise eine Beziehung zwischen der Rapssortenwahl und der Abundanz von Durchwuchsraps zu prüfen. Der Einsatz der molekularen Marker zeigte, dass die Durchwuchspflanzen bei der Felderhebung weitgehend korrekt erfasst wurden. Auf vier von sieben ausgewählten Feldern konnten die Pflanzen Liniensorten zugeordnet werden, die in den vorangegangenen Jahren angebaut worden waren. Auf zwei dieser Felder machte eine hoch dormante (HD) Liniensorte den

Großteil des Durchwuchses aus. Im Gegensatz trugen gering dormante Sorten kaum zum Raps-Durchwuchs bei. Hybridsorten konnten auf Grund der genetischen Aufspaltung in der F_2-Generation in keinem Fall identifiziert werden. Zusammengefasst weisen die Ergebnisse darauf hin, dass HD-Sorten wesentlich zur Abundanz von Durchwuchsraps auf landwirtschaftlichen Flächen beitragen, auch wenn dies noch durch zusätzliche Daten bestätigt werden muss.

Stichwörter: *Brassica napus*, Durchwuchsraps, ISSR-PCR, Samenüberdauerung, sekundäre Dormanz, Winterraps

1. Introduction

In 2009, german farmers produced 6.3 million t rapeseed (10.6 % of world rapeseed production) on about 1.5 million ha arable land (4.8 % of world rapeseed acreage) (SCHAACK et al., 2010). This reflects the high productivity of OSR cultivation driven by the worldwide increasing demand for edible oil and biofuels.

OSR cultivation implicates agronomic issues linked to the long-term seed persistence in soils and oilseed rape volunteer emergence in subsequent crops. OSR seeds can become dormant when exposed to darkness and water stress often caused by deep burial through tillage, and may then persist in the soil for long times. OSRV emerging in another OSR crop may harm product quality. For instance, double-low quality volunteers can affect the fatty acid composition of special varieties (e.g. high oleic acid, low linolenic acid; CLARKE et al., 2011). In the case of herbicide resistant (genetically modified (GM) or non-GM) OSR, volunteer management may be more challenging in subsequent crops. BECKIE et al. (2006) reported enhanced farmers´ awareness of volunteer problems 10 years after the introduction of herbicide resistant GM OSR in Canada. In the European Union, GM volunteers can cause exceeding of the labeling threshold (0.9 %) for GM admixtures in non-GM food and feed (MESSÉAN et al., 2007).

Previous studies revealed that land management impacts on OSRV abundances considerably. Significant factors are crop rotation (DEVOS et al., 2004), soil tillage (GRUBER et al., 2010) and variety selection (GRUBER et al., 2009; GULDEN et al., 2004; MOMOH et al., 2002). GRUBER et al. (2010) showed in field burial experiments that genotypic secondary dormancy variation resulted in a slower soil seed bank decline of a high-dormancy OSR variety compared to a low dormancy variety.

Therefore, an on-farm study was carried out to investigate the long-term factors for OSRV abundance. Field-specific data were surveyed including the OSR variety selection between harvest years 1997 and 2010 respectively 2011 in order to derive a prediction model on volunteer abundance. Preliminary data exploration revealed that OSR variety selection was elusive as a factor for statistical analysis due to the varying cropping histories of agricultural fields (THÖLE et al., 2011). Therefore an objective of our study was to assess the impact of OSR variety selection on volunteer abundance after having identified the genotypic origin of volunteers by DNA fingerprint analysis. In addition, the molecular analysis allowed a quality control of the survey methods by distinguishing OSRV from cultivated varieties.

2. Materials and methods

2.1 Plant material

OSRV were surveyed on winter OSR fields in Northern and Eastern Germany. As a precondition, OSR had to be grown at least once between harvest years 1997 and 2009 respectively 2010. Interviews with farmers provided information about the potential genetic origin of OSRV in the experimental fields, i.e. about previously cultivated OSR varieties. Two methods were used to survey OSRV abundances: in spring 2010, distinctly longer plants were assigned to OSRV in fields sown with the semi-dwarf OSR hybrid variety “Avenir” in autumn 2009 (Tab. 1). Alternatively in one field, OSRV were counted in autumn 2010 between wide rows (row spacing of 0.5 m). Leaf samples were collected from plants identified as putative volunteers. Leaf material of field-specific reference OSR varieties was obtained from plants cultivated in a greenhouse. OSR cropping histories and the frequencies of OSR cultivation since 1997 of selected fields are shown in Table 1.

2.2 ISSR-PCR

DNA was extracted from leaf samples using the NucleoSpin® Plant II extraction kit (Macherey-Nagel, Düren, Germany). 25 µl PCR reactions contained 25 mM $MgCl_2$, 2.5 mM of each dNTP, 1 u GoTaq® DNA polymerase (Promega), 25 ng template DNA and 15 pmol ISSR-primer. Seven different 3' and 5' anchored degenerate ISSR-primers according to CHARTERS et al. (1996) were obtained from Eurofins MWG Operon (Ebersberg, Germany). PCR amplifications were carried out in a Biometra T1 thermocycler (Biometra biomedizinische Analytik GmbH, Goettingen, Germany). PCR products were separated by electrophoresis on 1.5 % agarose gels. Gels were stained in ethidium bromide solution and photographed under UV light.

2.3 Cluster analysis

For cluster analysis, binary matrices reflecting the presence (1) and absence (0) of ISSR bands were generated. Bands were scored in the range of 300-2000 base pairs. Genetic distances were estimated by the Nei and Li coefficient (NEI and LI, 1979). The UPGMA method (unweighted pair group method with arithmetic mean) was used to draw dendrograms.

3. Results

The seven ISSR primers used for the study generated 87 reproducible bands of which 48 bands were polymorphic. In initial ISSR-PCR tests, reference OSR varieties were found to be sufficiently distinguishable.

3.1 Identification of OSR volunteers

Table 1 demonstrates that OSRV were mostly identified correctly in fields with the semi-dwarf variety. In fields B and C, 100 % of sampled plants proved to be true volunteers, i.e. did not cluster with the presently cultivated OSR variety. In contrast, in field F only 48 % of plants sampled were assigned to be volunteers. As a result of the molecular characterization, the volunteer abundance shown in Table 1 as median of OSRV per m^2 was corrected downwards for this field as well as for fields A, D and E. In field G, uniform and accurate seed placement at row distances of 0.5 m was provided by planters. In this field, 100 % of OSR plants between rows were identified as volunteers.

Tab. 1 Identification of oilseed rape volunteers (OSRV) and volunteer assignment to winter OSR varieties.
Tab. 1 *Klassifizierung von Durchwuchsraps und Zuordnung der Durchwuchspflanzen zu Winterrapssorten.*

Field	Winter OSR varieties grown in harvest years 1997-2011[1)]	OSR frequency in the rotation (%)	OSRV per m^2 (median)	Plants correctly identified as OSRV (%) [number of plants sampled]	Volunteer assignment to OSR varieties (%) [number of plants]
A	2005: Smart (OP, h) 2010: Avenir (H, SD)	8	3.5	89.5 [19]	Smart: 73.7 [14]
B	2003: Smart (OP, h) 2007: Elektra (H, m) 2010: Avenir (H, SD)	15	4.5	100 [22]	Smart: 45.0 [10]
C	2002: Artus (H, m) 2007: NK Fair (OP, l) 2010: Avenir (H, SD)	15 (since 1999)	0.5	100 [9]	NK Fair: 33.3 [3]
D	2002: Talent (H, m) 2005: SW Calypso (H, *) 2007: SW Calypso (H, *) 2010: Avenir (H, SD)	38 (since 2002)	10.0	89.7 [29]	Not determined
E	2005: Titan (H, l) 2010: Avenir (H, SD)	8 (since 2000)	1.5	71.4 [7]	0
F	2002: Express (OP, l) 2006: NK Fair (OP, l) 2010: Avenir (H, SD)	15	1.0	48.0 [25]	0
G [2)]	1999: Mohican (OP, h) 2004: Maplus (OP, l) 2007: ES Astrid (OP, *) 2011: PR46W20 (H)	21	2.5	100 [23]	ES Astrid: 73.9 [17]

[1)] Dormancy potential due to GRUBER et al. (2009): * unknown, l: low; m: medium; h: high; OP: open pollinator; H: hybrid; SD: semi-dwarf. [2)] Survey between wide rows (0.5 m).

3.2 Assignment of volunteers to OSR varieties

The results for the assignment of volunteers to previously cultivated varieties are field-specific. In four fields the genetic origin of volunteers could be reconstructed to a high degree. In fields A and B, the variety "Smart" with known high dormancy potential was identified predominantly (field A: 73.7 %, field B: 45.0 %). As Figure 1 shows exemplarily, 10 of 22 plants sampled from field B clustered very well with the reference samples of variety "Smart". In contrast, different plants of the hybrid variety "Elektra" showed only weak clustering, therefore hindering a reliable assignment of volunteers to this variety. In field G, 73.9 % of volunteers were assigned to "ES Astrid" with unknown dormancy potential. In field C, one third of the volunteers clustered with the recently grown low dormancy variety "NK Fair". ISSR-PCR analysis, however, revealed no identification of the genotypic origin of volunteers in fields E and F. In field D, OSR variety identification of volunteers was omitted because of the high OSR frequency and the cropping succession with different hybrid varieties (Talent, SW Calypso), which we assumed led to extensive hybridizations and genetic segregation of hybrid varieties in the F_2 generation.

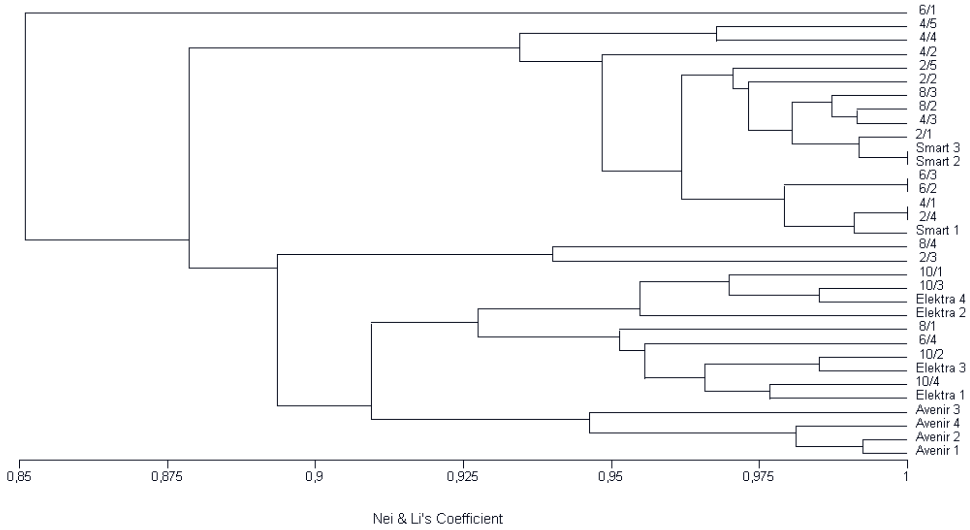

Fig. 1 Cluster analysis for winter OSR varieties and volunteers in field B (UPGMA). Individual volunteer plants are marked by double codes.

Abb. 1 *Clusteranalyse für Winterrapssorten und Durchwuchsraps auf Schlag B (UPGMA). Einzelne Durchwuchspflanzen sind durch Doppelcodes gekennzeichnet.*

4. Discussion

4.1 Identification of OSR volunteers

The results demonstrate that in most cases OSR volunteers could be distinguished successfully from a semi-dwarf hybrid OSR crop. Nevertheless, this finding is not universally valid, as is obvious from field F, where longer OSR plants were erroneously identified as volunteers. This is possibly due to the fact that varieties can vary in plant height due to environmental conditions (KOCH and KREYE, 2007). Therefore, longer plants of the semi-dwarf variety may have been unintentionally identified as volunteers. Especially, when volunteer sampling was conducted at the beginning of flowering as was the case for field F, differentiation between the semi-dwarf variety and volunteer plants was more difficult than during earlier growth stages.

Alternatively, OSRV can be surveyed in autumn in artificial sowing gaps (SÖCHTING et al., 2008) or between wide rows (0.5 m row spacing). Sowing gaps are produced by lifting the coulters of a seed drill while passing the field. In sowing gaps, errors in volunteer detection are possible as well (THÖLE and DIETZ-PFEILSTETTER, unpublished), especially because of seed losses dropping into the gaps from seed coulters. Survey methods can also be combined, provided that OSR winter survival is close to 100 %. This is useful to spread the workload of surveyors and to maximize the sample size. In spring, surveys in sowing gaps are feasible until OSR stem extension. From the start of stem extension until the start of flowering, volunteers can be detected in OSR semi-dwarf crop stands.

Altogether, the volunteer survey in sowing gaps seems to be preferable because less effort for farmers is necessary compared to sowing of semi-dwarf varieties where seed admixture with other OSR types might be undesirable. The success of detecting OSRV between wide rows cannot be finally assessed. For instance, ANDERSEN et al. (2010) collected OSR plants between wide rows of organic OSR fields and by ISSR-PCR confirmed the volunteer origin of these plants for two of five fields only. While seed isolation with planters is adopted increasingly in agricultural OSR cultivation practice, the latter method is promising because no additional efforts are requested from farmers.

4.2 Assignment of volunteers to OSR varieties

This study reveals that the assignment of volunteers to previously cultivated varieties was largely feasible on fields with low to medium OSR cropping frequency when open pollinating varieties had been grown. However, volunteers could never be assigned to hybrid varieties, most likely due to genetic segregation of hybrids in the F_2 generation. There was also one field (field F) where none of the volunteers could be allocated to previously grown open pollinating varieties. Similar drawbacks were also observed by JØRGENSEN et al. (2007) who suggested external sources like seed impurities or seed spillage as possible origins of non-identifiable plants.

In two of the fields, open pollinating varieties with high dormancy potential were found in very high proportions, contributing substantially to high volunteer abundance. A small number of low dormancy volunteers were identified in one field, originating from the most recently cultivated variety. A predominance of high dormancy varieties among volunteers is confirmed in another study where OSRV were counted in sowing gaps (THÖLE and DIETZ-PFEILSTETTER, unpublished).

No simple correlation, however, could be derived between volunteer abundance and the dormancy potentials of previously cultivated varieties – in contrast to findings in a study under controlled conditions (GRUBER et al., 2010). Under on-farm conditions, the impact of OSR genotype selection may be confounded by unrecognized and/or multiple interactions with other factors. Environment and land management practices are known to influence volunteer abundances. GRUBER et al. (2010) state that timing of first post-harvest tillage after OSR is very important. Under dry soil conditions, OSR seed incorporation into the soil is linked to second dormancy induction (LUTMAN et al., 2003). THÖLE et al. (2011) derived from a regression tree analysis that volunteer abundance under on-farm conditions is mainly attributable to locations and OSR cropping frequencies. WEBER et al. (2011) explain location effects by the soil texture, which was also suggested by LUTMAN et al. (2005).

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