Federal Biological Research Centre for Agriculture and Forestry[1]), Bayer Crop Science Deutschland GmbH[2])

Occurrence of transgenic progenies in the harvest of winter oilseed rape variety trials

Auftreten von transgenen Nachkommen im Erntegut von Wertprüfungen Winterraps

Antje Dietz-Pfeilstetter[1]), Walter Bübl[2]), Dieter Stelling[2])·[3])

Abstract

In order to determine the portion of transgenic, herbicide resistant progeny occurring in the non-transgenic harvest during the joint testing of genetically modified and conventional varieties of winter oilseed rape *(Brassica napus* L. *)*, we harvested variety trials at 12 different test sites. Two random samples per test site of the bulked seed material of the non-transgenic varieties were screened for seedlings resistant to glufosinate by a herbicide germination test, followed by a PCR analysis of resistant seedlings. The average rate of resistant seedlings was 0.53 % with a range of 0.08 to 1.16 %. The portion of transgenic seeds differed in part considerably between samples of the same seed lot. Possible reasons for this variation are discussed.

Key words: Transgenic plants, sampling, adventitious presence, oilseed rape, pollen flow

Zusammenfassung

Um den bei der gemeinsamen Wertprüfung von gentechnisch veränderten und konventionellen Sorten von Winterraps *(Brassica napus* L.*)* im nicht-transgenen Erntegut auftretenden Anteil von transgenen, herbizidresistenten Nachkommen zu bestimmen, wurden Sortenprüfungen von 12 verschiedenen Standorten geerntet. Zwei Zufalls-Stichproben wurden pro Standort aus dem Gesamterntegut der nicht-transgenen Sorten mittels eines Herbizid-Keimungstests auf Keimlinge mit Resistenz gegen Glufosinat untersucht, gefolgt von einer PCR-Analyse der resistenten Keimlinge. Der durchschnittliche Anteil resistenter Keimlinge betrug 0,53 %, wobei die Rate zwischen 0,08 % und 1,16 % schwankte. Der Anteil transgener Samen war bei den beiden Stichproben desselben Gesamternteguts zum Teil sehr unterschiedlich. Mögliche Ursachen für diese Variation werden diskutiert.

Stichwörter: Transgene Pflanzen, Probenahme, Saatgut-Beimengung, Raps, Pollenflug

Introduction

Pollen-mediated gene flow between oilseed rape fields can occur over long distances (SCHEFFLER et al., 1993; HALL et al., 2000; RIEGER et al., 2002) and cannot be prevented even by planting border areas of synchronously flowering non-transgenic oilseed rape (STANILAND et al., 2001). It is therefore expected, that in plot trials of genetically modified and conventional, non-transgenic varieties of winter oilseed rape, outcrossing of the transgenic traits into plants of the conventional plots will occur. The proportion of outcrossed seed containing the transgene is determined by: the relative number of transgenic plants; the sensitivity of the conventional rape varieties to cross pollination; the placement, size and distance of the plots (SCHEFFLER et al., 1993; LEVIN and KERSTER, 1974); the overlapping of the flowering periods; weather conditions; pollinator activity and other factors.

The aim of the current study was to determine the portion of transgenic seeds in the total harvest of non-transgenic varieties in a series of twelve oilseed rape variety trials in Germany.

Materials and Methods

Plant materials and field design

In the growing season 1999/2000 the German plant variety authority „Bundessortenamt" tested six genetically modified oilseed rape varieties (one open-pollinated and five hybrid varieties) within the official variety testing series for winter oilseed rape at 15 locations within Germany. All transgenic varieties were resistant to glufosinate, the herbicidal compound of the herbicides Basta® and Liberty®. The open-pollinated variety was derived from the transformant GS40/90 (homozygous for the *pat*-gene; *pat/pat*), whereas the other five varieties were fully restored hybrids, three of which were based on the transformants Ms8/Rf3 (i.e. the hybrid system SeedLink® which is linked with the Liberty resistance (homozygous for the *bar*-gene; *bar/bar*)). The remaining two hybrids were derived from either transformant L62 (heterozygous for the *pat*-gene; *-/pat*) or transformant GS40/90 (heterozygous for the *pat*-gene; *pat/-*). The conventional assortment encompassed altogether 82 varieties, 48 of which were open-pollinated lines, 32 were fully restored hybrids and 2 were varietal associations. In addition, several border and buffer plots were planted with conventional oilseed rape varieties.

Varieties were separated into three sub-groups according to the type of variety (open-pollinated lines, fully restored hybrids and varietal associations). The experimental layout was a split-plot-design with four replications. Most of the trials were laid out with double plots; only at four of the 12 harvested test sites were single plots sown. The size of the plots varied be-

---

[3]) at present Deutsche Saatveredelung Lippstadt-Bremen GmbH

tween 12 and 27 m². The official variety trial was partly surrounded by other field trials or agricultural fields with transgenic or non-transgenic oilseed rape, as indicated in Table 2.

Harvesting and analysis of the harvested seeds

At 12 of the 15 variety test sites trials were harvested. Seeds from the plots grown with genetically modified varieties and seeds from those grown with conventional varieties were harvested separately. From the dry and cleaned bulked harvest seed material of the non-transgenic varieties (with seed quantities ranging from 1500 to 5400 kg per test site) representative test samples of 1000 g were taken by a sworn expert. Two randomly chosen samples per test site were subsequently screened for transgenic seeds by analysing 2000 seeds from each sample; for the test site Böhnshausen 4000 seeds were tested per sample.

Germination test

The portion of seedlings resistant to glufosinate was determined according to the germination test developed by PFEILSTETTER et al. (2000). 1000 seeds were placed in a transplanting tray on filter paper soaked with an aqueous solution of 0.005 % Basta®. After 10 days seedlings which developed were examined. For verification, seedlings classified as resistant or probably resistant were potted into soil and subsequently subjected to a PCR analysis.

PCR analysis

For PCR amplification, DNA from plant leaves was isolated according to TINKER et al. (1993) using CTAB extraction buffer (2 % cetyltrimethylammonium-bromide (w/v), 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 0.1 M Tris-HCl pH 8.0). 100 ng of DNA was amplified in separate reactions using either the *pat* gene-specific primer combination CaMV-F/Pac3-R described by PFEILSTETTER et al. (2000) or primers for the *bar* gene amplification (bar3, forward, 5′-GAAC-CGCAGGAGTGGGAC-3′ and bar2, reverse, 5′-GGCAGCCC-GATGACAGC-3′). Reactions (50 µl) containing 1x standard PCR reaction buffer, 20 pmol of each primer, 100 µM dNTPs, 1 % DMSO (dimethyl sulfoxide) and 1 unit Taq polymerase were incubated in a TC1 thermocycler (Perkin Elmer) for 35 cycles af-

ter an initial denaturation at 94 °C for 2 min: 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C. 15 µl of PCR products were separated by electrophoresis on a 1 % agarose gel.

Results

The seed lots harvested from the different trial sites showed with one exception (Sickte) medium to high germination rates (72 % – 97 %, see Table 1). The germination rates were taken into consideration when calculating the portion of transgenic rape seeds. Adjusted for the number of germinated seeds and as identified by the herbicide germination test, the medium portion of resistant seedlings in the 24 samples of the conventional seed lots from 12 test locations was 0.53 % (Table 1); three samples had values above 1 %. From the twelve different test locations, one harvest had an average transgenic seed portion of above 1 %, five were between 0.5 % and 1 % and the remaining six were below 0.5 %. The variance of the sampling error proved to be higher than that of the different seed lots (ANOVA not shown).

In the following PCR analysis 95.1 % of the 184 investigated resistant or probably resistant seedlings proved to be transgenic.

Discussion

The official variety testing trials contained 82 conventional varieties and six genetically modified oilseed rape varieties. Four of the 6 genetically modified varieties were homozygous for the *pat* or the *bar* gene, while two were heterozygous for the *pat* gene. Taking into account additional buffer zones planted with non-transgenic oilseed rape varieties, it can therefore be calculated, that due to the genetic structure and the portion of transgenic varieties less than 5.3 % of the total oilseed rape pollen cloud should have been transgenic. Assuming an average interplant outcrossing rate of about 25 % (RAKOW and WOODS, 1987; PERSSON, 1956) and optimal conditions for cross pollination, the share of transgenic seeds within the harvest of the conventional varieties was expected to be not more than 1.33 %.

The observed portions of transgenic seeds were consistently below this value, probably in part due to additional pollen coming from conventional oilseed rape varieties planted in the sur-

Table 1. Occurrence of genetically modified seeds in the harvested seed bulk of conventional varieties tested in the official variety trials for winter oilseed rape in 1999/2000 in Germany. Genetically modified seeds were determined by germination test on 0.005 % Basta® solution and subsequent PCR analysis (see Mat. and Meth.)

Test site	Germination rate (%)	Portion of resistant and probably resistant seedlings (%)**	Portion seedlings with *pat.* or *bar* gene, resp. (%)
Solschen	97	0.08/ –	0.05*
Böhnshausen	90	0.05/0.03	0.08
Seligenstadt	88	0.17/0.06	0.23
Biemsen	92	0.24/ –	0.24*
Reussenköge	92	0.28/0.06	0.30*
Boldebuck	84	0.40/0.03	0.42*
Kl. Wesenberg	72	0.53/0.11	0.49*
Adelshausen	88	0.57/0.18	0.69
Kleptow	77	0.64/0.10	0.71*
Malchow	96	0.63/0.11	0.74
Zolling	83	0.88/0.12	0.96*
Sickte	43	0.93/0.23	1.16*
Average	**83.3**	**0.45/0.08**	**0.50**
Standard variation	±14.6	±0.29/±0.07	±0.35
Standard error s_e	4.4	0.22/0.06	0.22
L.S.D.$_{.5\%}$***	13.8	0.68/0.18	0.69

* between 1 and 3 seedlings each died before PCR testing; assumption that seedlings not analysed in the PCR also contain the *pat* or *bar* gene.
** The second number represents seedlings phenotypically not unambiguously classified as resistant in the germination test.
*** L.S.D.$_{.5\%}$: least significant difference at 5 % error probability.

Table 2. Description of the test sites of the official variety trial series for winter oilseed rape in the growing season 1999/2000 in Germany, from which seeds were harvested

Test site	Plot size (m²)	Plot shape*	one-, two-, three- or four-sided	Surrounding winter oilseed rape fields are conventional transgenic Distance to variety trial (m)	one-, two-, three- or four-sided
Solschen	18.0	DL	2	≥100	No
Böhnshausen	14.1	DL	2	≥100/≥500	No
Seligenstadt	25.0	DL	3	≥10	No
Biemsen	18.0	DL	4	≥10	No
Reussenköge	24.0	DL	3	≥10	No
Boldebuck	12.0	SL	1	≥100	No
Kl. Wesenberg	11.2	SL	3	≥10	No
Adelshausen	18.0	DL	none	>1000	No
Kleptow	27.0	DL	1	≥200	No
Malchow	22.5	DL	1	≥10	No
Zolling	18.0	SL	3	≥10/≥500	No
Sickte	12.0	SL	2	≥100/≥500	1 (≤100 m)

* DL: double plot, SL: single plot with the exception that only six short varieties tested in double plots.

rounding area (see Table 2). Other explanations for the observed low values could be the unequal spatial distribution of the transgenic plots in the trial and/or possibly lower visible outcrossing rates at the plot level (HÜHN and RAKOW, 1979). The tendency for higher portions of transgenic seeds at locations with mostly single plots indicates, that pollinations from other varieties are favoured, when plots are smaller and longer. In the larger double plots with a more square shape, however, the probability of pollinations between plants within the same plot is enhanced, i.e. a greater portion of the "outcrossing" pollen comes from plants of the same variety. And this will be even more true in commercial large-scale oilseed rape crop stands.

The highest portion of transgenic seeds was observed at the location Sickte which, interestingly, was the only test site, where another field trial with transgenic herbicide resistant oilseed rape was located close to the investigated variety trial (Table 2).

It has to be pointed out, however, that the results of the present investigation are probably charged with a considerable sampling error. The average difference between transgenic seed portions in independent samples of the same location was 0.27 %, with variation ranging between 0.00 and 1.06 %. Sampling errors might be caused by heterogeneities of the harvest material as well as by irregularities in the sampling and/or processing of the test samples (KRUSE, 2002).

Heterogeneities of the harvest material may be due to: the unequal distribution of the plots with genetically modified varieties within the test field; differences in the pollen flow dependent on the direction of the wind and/or the presence of insects; or variations in a successful pollination by foreign pollen. DIEPENBROCK et al. (2001) demonstrate that indeed the variation between samples from differently located plots can be very high. In addition, these authors found that the type of variety has a significant effect on the portion of transgenic progenies. The tested composite hybrid variety consisting of a varietal association with 80 % male sterile hybrid plants and only 20 % male fertile plants had a considerably higher rate of cross pollination than the tested fertile open-pollinated varieties and restored hybrids, thus confirming that sterile plants are frequently better pollen receivers than fertile plants. A higher susceptibility of a composite hybrid variety to foreign pollen is also reported by SIMPSON et al. (1999). Other factors, that may contribute to heterogeneous harvest samples in the present study, are the lack of mixing of the harvested seed material from the different plots and possible carry overs during the harvest.

It has to be stressed, that the percentages of transgenic seeds in conventional seed lots from variety trial sites may not be ex-

tended to commercial oilseed rape fields. The data recently published by RIEGER et al. (2002) on large-scale oilseed rape fields in Australia indicate, that even in adjacent commercial fields the portion of outcrossed seeds from single field sources will be well below 1 %.

The high portion of positive PCR signals (95.1 %) for the transgenes (*pat* and *bar* gene) observed for the plants positively selected for glufosinate resistance in the herbicide germination test confirms earlier findings of PFEILSTETTER et al. (2000) on the suitability and diagnosis power of this germination test. Whether quantitative PCR methods testing seed bulks deliver similar or even better data and may replace screening methods testing single seeds has to be verified.

References

DIEPENBROCK, W., C. SCHUSTER, K. FÖRSTER, A. SCHUBERT, 2001: Mehrortige Untersuchungen zu den ökologischen Auswirkungen des Anbaus von herbizidresistentem Raps auf die Flora von Ackerflächen und Ruderalstandorten in Sachsen-Anhalt. Forschungsbericht Nr. 76213/45/97H im Auftrag des LAU Sachsen-Anhalt.
HALL, L., K. TOPINKA, J. HUFFMANN, L. DAVIS, A. GOOD, 2000: Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple-resistant *B. napus* volunteers. Weed Science **48**: 688–694.
HÜHN, M., G. RAKOW, 1979: Einige experimentelle Ergebnisse zur Fremdbefruchtungsrate bei Winterraps *(Brassica napus oleifera)* in Abhängigkeit von Sorte und Abstand. Z. Pflanzenzüchtg. **83**: 289–307.
KRUSE, M., 2002: Die Bedeutung der Saatgutprobenahme bei GVO-Schwellenwerten nahe 0%. In Deutsche Forschungsgemeinschaft: Schwellenwerte für Produkte aus gentechnisch veränderten Pflanzen. Wiley VCH Verlag, Weinheim. Mitteilung **7**, pp 72–79.
LEVIN, D. A., H.W. KERSTEN, 1974: Gene flow in seed plants. Evolutionary Biology **7**: 139–220
PERSSON, B., 1956: Investigations concerning insect pollinated crops. III. The importance and extent of intercrossing in rape *(Brassica napus* L.) with special reference to the activity of honeybees. Statens Vaxtskyddsanst. Medd. **70**: 1–36.
PFEILSTETTER, E., A. MATZK, S. D. FELDMANN, J. SCHIEMANN, 2000: Rapid and efficient screening of phosphinothricin tolerant oilseed rape *(Brassica napus)* with a novel germination test. Euphytica 113: 119–124.
RAKOW, G., D. L. WOODS, 1987: Outcrossing in rape and mustard under Saskatchewan prairie conditions. Can. J. Plant Sci. **67**: 147–151.
SCHEFFLER, J. A., R. PARKINSON, P. J. DALE, 1993: Frequency and distance of pollen dispersal from transgenic oilseed rape *(Brassica napus)*. Transgenic Res. **2**: 356–364.

RIEGER, M. A., M. LAMOND, C. PRESTON, S. B. POWLES, R. T. ROUSH, 2002: Pollen-mediated movement of herbicide resistance between commercial canola fields. Science **296**: 2386–2388.

SIMPSON, E. C., C. E. NORRIS, J. R. LAW, J. E. THOMAS, J. B. SWEET, 1999: Gene flow in genetically modified herbicide tolerant oilseed rape (*Brassica napus* in the UK. In P Lutman, ed, Gene Flow and Agriculture – Relevance for Transgenic Crops, British Crop Protection Council, University of Keele, Staffordshire UK, pp 75–82.

STANILAND, B. K., P. B. E. McVETTY, L. F. FRIESE, S. YARROW, G. FREYSSINET, M. FREYSSINET, 2001: Effectiveness of border areas in confining the spread of transgenic *Brassica napus* pollen. Can. J. Plant Sci. **80**: 521–526.

TINKER, N. A., M. G. FORTIN, D. E. MATHER, 1993: Random amplified polymorphic DNA and pedigree relationships in spring barley. Theoretical and Applied Genetics **85**: 976–984.