ANNEX 4

2012 MONITORING FOR VOLUNTEER POTATOES AT 2010 STARCH POTATO PRODUCTION FIELDS

BASF

The Chemical Company

BASF Plant Science

BASF STUDY NUMBER # AP-527-13-001

2012 MONITORING FOR VOLUNTEER POTATOES AT 2010 STARCH POTATO PRODUCTION FIELDS

DATA REQUIREMENT: N/A

AUTHOR:

[*name deleted*]

STUDY INITIATED ON: MAY 15, 2012

STUDY COMPLETED ON: MARCH 13, 2013

TEST FACILITIES:

AMFLORA 2010 STARCH PRODUCTION FIELDS,
CZECH REPUBLIC, AND

PERFORMING LABORATORY:

SUNGENE GMBH, A BASF PLANT SCIENCE COMPANY
CORRENSSTR. 3
06466 GATERSLEBEN
GERMANY

SUBMITTED BY:

BASF PLANT SCIENCE COMPANY GMBH
CARL-BOSCH-STR. 38
D-67056 LUDWIGSHAFEN
GERMANY

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

Company: BASF Plant Science Company GmbH

Company Agent: [*name deleted*] Date: _____

Title: Regulatory Compliance Manager Europe

Signature: [*signature deleted*]

These data are the property of BASF Plant Science Company GmbH and, as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute in any other country.

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

The study described in this volume was not conducted in compliance with the OECD Principles of Good Laboratory Practice or the GLP Principles of German Chemikaliengesetz (Chemicals Act) and does not meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 150 (FIFRA)]. The data generated by BASF Plant Science Company GmbH in support of product safety comply with generally accepted scientific procedures. Record keeping is consistent with procedures used throughout the research community. This report accurately presents the raw data developed during the studies.

STUDY MANAGER:

[*name deleted*] _____ _____

Date

Sales Representative in Agro CZ
BASF spol s.r.o., AGRO
Sokolovská 668/136d
186 00 Praha 8, Czech Republic

SUBMITTER AND SPONSOR REPRESENTATIVE:

[*name deleted*] _____ _____

Date

Regulatory Compliance Manager Europe
BASF Plant Science Company GmbH
Carl-Bosch-Str. 38
D-67056 Ludwigshafen, Germany

CERTIFICATION OF AUTHENTICITY

We, the undersigned, hereby declare that this study was performed under our supervison according to the procedures described herein, and that this report provides a true and accurate record of the results obtained.

STUDY MANAGER:

[*name deleted*] _____ _____

Date

Sales Representative in Agro CZ
BASF spol s.r.o., AGRO
Sokolovská 668/136d
186 00 Praha 8, Czech Republic

SUBMITTER AND SPONSOR REPRESENTATIVE:

[*name deleted*] _____ _____

Date

Regulatory Compliance Manager Europe
BASF Plant Science Company GmbH
Carl-Bosch-Str. 38
D-67056 Ludwigshafen, Germany

STEWARDSHIP QUALITY MANAGEMENT STATEMENT

Study No.: AP-527-13-001

Study Title: 2012 MONITORING FOR VOLUNTEER POTATOES AT 2010 STARCH POTATO PRODUCTION FIELDS

BASF GB Regional Quality Manager, a member of the Stewardship Quality Management team, has inspected the Study Plan, Study File, and Final Report for compliance with the requirements for the post-market environmental monitoring of amylopectin potato EH92-527-1, variety Amflora.

[*name deleted*] _____ _____

Regional Quality Manager (or delegate) Date

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND DEFINITIONS

CFR	Code of Federal Regulations (USA)
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
gDNA	genomic DNA
IP System	Amflora Identity Preservation System
PCR	Polymerase chain reaction
qPCR	quantitative Polymerase chain reaction

STUDY INFORMATION PAGE

BASF Study Number	AP-527-13-001
BASF REG. DOC. NO.	2013/7000592
Study Title:	2012 Monitoring for Volunteer Potatoes at 2010 Starch Potato Production Fields
Sponsor:	BASF Plant Science Company GmbH Carl-Bosch-Str. 38 D-67056 Ludwigshafen Germany
Sponsor Representative	[*name deleted*]
Testing Facilities	Amflora 2010 starch production fields, Czech Republic
Study Manager	[*name deleted*]
Performing Laboratory	SunGene GmbH, Correnssstrasse 3 D-06466 Gatersleben Germany
Analysis Request Initiator	[*name deleted*]
Analysis conducted by	[*name deleted*]
Study Initiation Date:	May 15, 2012
Study Completion Date:	March 13, 2013
Location of Raw Data and Report	GLP archive, BASF Agricultural Center, D-67117 Limburgerhof, Germany

2012 MONITORING FOR VOLUNTEER POTATOES AT 2010 STARCH POTATO PRODUCTION FIELDS

SUMMARY

The amylopectin potato EH92-527-1, variety Amflora, has been genetically modified for increased amylopectin content in the tuber starch via transformation with a gene fragment encoding granule bound starch synthase (*gbss*) from potato in antisense orientation. This modification leads to the silencing of the amylose synthesizing enzyme in the potato tuber. In March 2010, Amflora was approved for commercial cultivation in the European Union and was grown for starch potato production at locations in the Czech Republic in 2010.

As part of the Amflora post-market environmental monitoring plan the purpose of this study was to evaluate the presence and persistence of Amflora volunteer plants and their frequency in the years following the Amflora cultivation for starch production.

Out of seven fields in the Czech Republic monitored in the second year following Amflora cultivation only at one field planted with maize two volunteer plants were detected. These two potato volunteers were confirmed as being Amflora potato plants.

INTRODUCTION

The amylopectin potato EH92-527-1, variety Amflora, has been genetically modified for increased amylopectin content in the tuber starch and was approved for commercial cultivation in the European Union in March 2010. Cultivation for starch potato production took place in the Czech Republic in 2010.

According to the Amflora post-market environmental monitoring plan the purpose of this volunteer monitoring study was to evaluate the presence and persistence of Amflora volunteer plants and their frequency in the years following the Amflora cultivation for starch production.

At harvest of potatoes always a certain portion of potato tubers remain in the field. The survival of these remaining potato tubers depends on soil management practices and

low temperature during the winter period. Tubers which survive under winter conditions can give rise to potato plants, also known as tuber-borne volunteer potatoes, in subsequent crops.

The purpose of this study was to demonstrate that amylopectin potato EH92-527-1 is comparable to conventional potatoes with regard to its competitive behavior and the capacity to survive or tolerate environmental conditions like frost. Evidence should be given that amylopectin potato EH92-527-1 does fit in the management scheme of conventional starch potatoes and that possible volunteer potatoes will be controlled effectively by the applied cultural practices.

Annex 2 of the Amflora post-market environmental monitoring plan (EU Register, 2010) outlines the number of production sites to be selected for surveillance should be 20. Therefore all Amflora starch production fields from 2010 were included in this volunteer monitoring study which relates to seven fields in the Czech Republic.

In addition to this volunteer monitoring study the volunteer monitoring according to the requirements set out by the Amflora Identity Preservation (IP) System was conducted at all sites where EH92-527-1 potato was grown in 2010.

MATERIALS AND METHODS

Potato volunteer monitoring. Monitoring was performed at all fields which were cultivated for Amflora starch production in 2010. This comprised a total of seven fields in the Czech Republic (Table 1). The monitoring was conducted at two time points during the cultivation period in 2012. The method described has been adapted from field inspection procedures and has already been used successfully for monitoring releases of genetically modified plants (MacDonald and Rouan, 2000).

Two different methods were applied to perform the volunteer monitoring: monitoring within the fields and outside the fields.

Volunteer monitoring within the field. Per field two to four points were chosen along one side of the field and marker stakes were driven into the ground to mark these points. Points were chosen e.g. along tractor lines passing the field to facilitate walking through the field for the observer. GPS-coordinates of the points were taken and recorded. Vertical lines through the field were mapped out and the observer walked along these lines across the field (blue and green lines in Figures 1, 4, 8, 11, 14, 17, and 20). Per mapped vertical line six to 10 plots were selected randomly to a total number of 20 observation plots per field. The individual selected plots were at least 5 m apart from each other and the six to 10 plots were distributed randomly across the field along the mapped vertical line. As recommended by Roberts-Pichette and Gillespie (1999) for each plot an area of 1 m x 1 m was measured and this square meter represented the area to be monitored for the occurrence of potato volunteers. The number of observed volunteers within the 1 m^2 plots was recorded. For any volunteer potatoes found, one leaf was taken and analyzed for identity via PCR analysis. After recording the volunteer occurrence and in order to destroy the observed volunteers according to the requirements of the Amflora IP System, they were dug out of the soil and left on the field for composting.

Volunteer monitoring outside the field. The observer monitoring for the occurrence of tuber-borne Amflora volunteers walked around the circumfence of the field at a distance of 1 m to the outer edge of the area cultivated with Amflora in 2010. Thereby, an area of 1 m left and 1 m to the right (resulting in a 2 m wide stripe directly surrounding Amflora area) was monitored for the presence of potato volunteers. The number of volunteers was recorded and the area where the volunteers were found marked in the map. For the two volunteer potatoes found, leaf samples were taken and analyzed for identity via PCR analysis. Following the Amflora IP System, observed volunteers were dug out of soil in order to destroy the potato volunteers.

PCR Analysis. Leaf samples from the volunteer potatoes were taken in the field, put in bags and transported to the test facility SunGene GmbH, Gatersleben, Germany. At SunGene (MA Request SG12_243), the leaves from the two plants (sample numbers TS-PRE-PPH1-1-3680944 and TS-PRE-PPH1-1-3680945) were divided into two samples each for analysis. These four samples (TS-PRE-PPH1-1-3680944[1], TS-PRE-PPH1-1-3680944[2], TS-PRE-PPH1-1-3680945[1], TS-PRE-PPH1-1-3680945[2]) were analyzed individually by a validated real-time PCR measurement (SG_Assay-0654).

DNA Extraction. For each sample, a small piece (approximately 20 mg) of the leave was taken. Samples were frozen lower than -65°C and homogenized by grinding for 30sec at 30 Hz in a Retsch mill according to the protocol "DNA Isolation aus Blättern unter Verwendung des Wizard Magnetic 96 DNA Plant System (Promega), SOP: SG/MA 0001/2008 Version 007. The homogenized material was lysed in Lysis Buffer A and thoroughly shaked. After centrifugation, the clear supernatant was mixed with MagneSil Paramagnetic Particles, FF377X, (Wizard Magnetic 96 DNA Plant System, FF3761X). Subsequent to a 5 minute incubation step, the DNA bound to the magnetic beads was washed 3x by placing and displacing the 96 well flat-bottom plate onto a magnet. After washing and drying of the magnetic beads, genomic DNA bound on magnetic beads was eluted with Millipore water. Two independent samples were isolated and the results obtained from the duplicate samples were equivalent.

DNA Quantification. The concentration of gDNA isolated by this method is on average around 20ng/µl, and was thus not determined for each individual sample. As duplex qPCR reactions were performed, the presence of a typical DNA amount in each individual sample was confirmed by the Ct value of qPCR reaction of the endogenous reference gene.

Quantitative Real-time PCR Setup. TaqMan® assays (Applied Biosystems, Carlsbad, CA USA) were performed in a 96 well plate format on an ABI PRISM® 7500 sequence detection system (Applied Biosystems) using a BASF Plant Science internally validated event-specific real-time qPCR TaqMan® assay (SG_Assay-0654).

Assay conditions and primers and probe sequences were as described in JRC (2009). All analyses were performed as duplex PCR assays using an endogenous potato gene as reference. As these assays were performed as duplex reactions, while the JRC (2009) method is based on a simplex reaction, an alternate potato endogenous reference gene was used to generate this data (starch branching enzyme, StSBE1).

Raw data of qPCR analysis are the threshold cycles (Ct) as the point where the instrument first detects fluorescence above the background. The Cts for both the event-specific target and the potato endogenous reference gene StSBE1 for each pool were determined. A delta Ct value (dCt), representing the difference between the Ct value for the EH92-527-1 event assay and the Ct value for the endogenous reference gene, was calculated for each sample. Results for the presence of the EH92-527-1 event in each sample were calculated as either positive or negative based on the dCt value for each sample.

Controls. Each 96 well PCR plate included a 5-stage dilution series of the gDNA standard St-EH92(cn1)-001 from the EH92-527-1 event as positive control, one Prevalent wild type as a negative control for the EH92-527-1 amplicon and one well without any DNA template as a negative control for the reference endogene amplicon.

RESULTS AND DISCUSSION

Number of volunteers observed within and outside the field. A total of seven Amflora production fields in the Czech Republic were monitored for the occurrence of potato volunteers. Figures 1 to 22 illustrate the monitoring methodology as applied in 2012. During the second year following the cultivation of Amflora the monitoring was conducted in June and August 2012. The results of the observations for volunteer potatoes are summarized in Table 1. Only at one field (CZ02) a total of two volunteer potato plants were observed within the cultivated area (Table 1, Figures 6 and 7). These plants were found at the second monitoring time point in August, while passing the field, within close proximity to each other. Therefore it was decided to position a monitoring plot at this location. Leaf samples were taken for further analysis, however it was not possible to locate the mother tuber in the soil from which the potato volunteers developed. Most likely the mother tuber was buried in deep soil.

A plausible reason for the occurrence of volunteers within the field CZ02 might be that growing conditions for potato volunteers are more favorable in a maize field than within an oilseed rape or barley field where the emerging potato plant would get less light compared to a late developing maize field.

Outside the seven cultivation fields no potato plants were found.

PCR Analysis. The qPCR analysis from leaf samples taken from the two volunteer potatoes found confirmed that all samples taken derived from EH92-527-1 potatoes.

Isolated gDNA from samples of EH92-527-1 potato leaves was analysed using an EH92 527-1 event-specific duplex qPCR detection method. The qPCR raw data were calculated and a dCt value was obtained for each sample. The undiluted standard sample positive for the presence of the EH92-527-1 insert showed a dCt value of -0,4 compared to the reference gene, whereas the dCt value for the negative control sample was +15. The dCt values -0,3 to -0,6 allowed positive confirmation on the identity of the Amflora potato samples.

CONCLUSIONS

The current study provides evidence that in most cases (six out of seven fields) potential potato volunteer plants have been destroyed completely by soil management practices and by frosts following the Amflora cultivation in 2010. At the first monitoring time point in June 2012 no potato volunteer plants were detected neither within the fields nor in their surroundings. In August 2012 only two potato volunteer plants were found within the cultivated area of field CZ02.

Compared with the results from the first year of monitoring after Amflora cultivation the number of potato volunteers found had strongly decreased. In 2011, a total number of 66 potato volunteer plants were detected during the conduct of the monitoring study. This clearly demonstrates that the persistence of Amflora tubers does not differ from any other potato variety and that the cultivation and management practices applied during and following Amflora cultivation are appropriate to manage the potential survival of Amflora tubers remaining in the soil after harvest. Though it is noted that Amflora tubers surviving the soil treatment and frost period find more favorable conditions in a maize field to emerge compared to other crops used in rotation with potato like wheat, barley, or oilseed rape. In both years, 2011 and 2012, of monitoring after Amflora cultivation volunteer potatoes were only detected in those fields where the following crop was maize.

These findings confirm the information presented in Amflora Notification C/SE/96/3501 according to Directive 2001/18/EC (EFSA, 2006) and verify the assumption made in the environmental risk assessment.

REFERENCES

Carlson, J. E., Tulsieram, L. K., Glaubitz, J. C, Luk, V. W. K., Kauffeldt, C., and Rutledge, R. (1991) Segregation of random amplified DNA markers in F1 progeny of conifers. Theor. Appl. Genet. 83:194-200.

EFSA (2006) Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-UK-2005-14) for the placing on the market of genetically modified potato EH92-527-1 with altered starch composition, for production of starch and food/feed uses under Regulation (EC) No 1829/2003 from BASF Plant Science. EFSA Journal 324, 1-20. Available online: http://www.efsa.europa.eu/de/efsajournal/pub/324.htm

EU Register (2010) Post-market monitoring plan for Notification C/SE/96/3501. Available at: http://ec.europa.eu/food/dyna/gm_register/monitoringplan_eh92-527-1.pdf

JRC (2009) Event-specific method for the quantification of amylopectin potato event EH92 527 1 using real-time PCR. Available at: http://gmo-crl.jrc.ec.europa.eu/summaries/EH92-527-Validated_method.pdf

MacDonald, R. and Rouan, D. (2000) Monitoring commercial releases of genetically modified crops: InVigor Hybrid Canola. The 6th International Symposium on The Biosafety Of Genetically Modified Organisms, July 2000, Saskatoon, Canada.

Roberts-Pichette, P. and Gillespie, L. (1999) Terrestrial vegetation biodiversity monitoring protocols. EMAN Occasional Paper Series, Report No. 9. Ecological Monitoring Coordinating Office, Burlington, Ontario.

Table 1. Number of potato volunteers found 2012

				Number of volunteers observed June 2012		Number of volunteers observed August 2012	
Field code	**Region**	**Field size [ha]**	**Crop planted in 2012**	**within the field**	**outside the field**	**within the field**	**outside the field**
CZ01	Olešná	33.6	peas and other feed crops	0	0	0	0
CZ02	Olešná	1.0	maize	0	0	2	0
CZ03	Olešná	11.5	peas and other feed crops	0	0	0	0
CZ04	Bohdalec	18.4	spring barley	0	0	0	0
CZ05	Bohdalec	28.4	maize	0	0	0	0
CZ06	Nové Dvory	2.0	oilseed rape	0	0	0	0
CZ07	Nové Dvory	44.1	oilseed rape	0	0	0	0

Figure 1. Fields CZ01 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the fields to select the observation plots in June and August 2012 respectively.

Figure 2. Field CZ01 – picture from volunteer monitoring
Field CZ01 and field surroundings at time of first volunteer monitoring in June 2012.

Figure 3. Field CZ01 – picture from volunteer monitoring
Field CZ01 at time of second volunteer monitoring in August 2012 conducted after harvest of the cultivated feed crops.

Figure 4. Field CZ02 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 5. Field CZ02 – picture from volunteer monitoring
Field CZ02 at time of first volunteer monitoring in June 2012.

Figure 6. Field CZ02 – picture from volunteer monitoring
Field CZ02 at time of second volunteer monitoring in August 2012.

Figure 7. Field CZ02 – picture from volunteer monitoring
Potato volunteer plant within the field CZ03 in August 2012.

Figure 8. Field CZ03 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 9. Field CZ03 – picture from volunteer monitoring
Field CZ03 at time of first volunteer monitoring in June 2012.

Figure 10. Field CZ03 – picture from volunteer monitoring
Field CZ03 at time of second volunteer monitoring in August 2012 conducted after harvest of the cultivated feed crops.

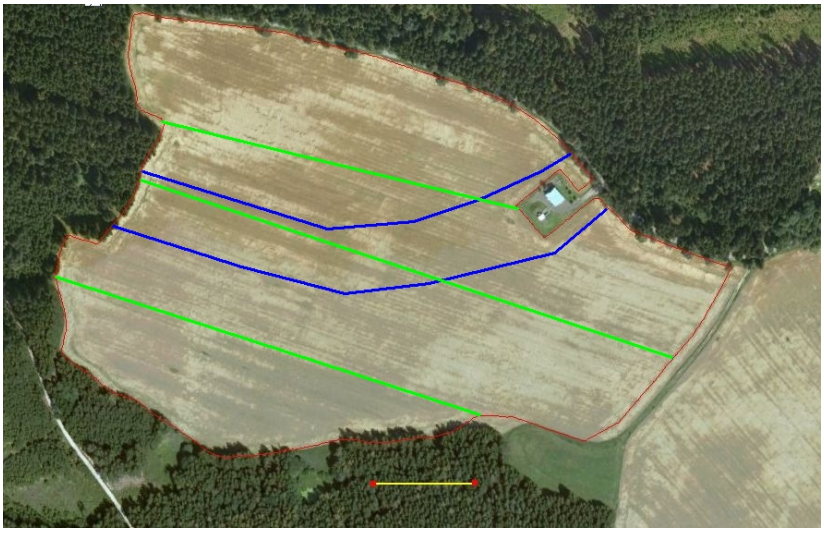

Figure 11. Field CZ04 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 12. Field CZ04 – picture from volunteer monitoring
Field CZ04 at time of first volunteer monitoring in June 2012.

Figure 13. Field CZ04 – picture from volunteer monitoring
Field CZ04 at time of second volunteer monitoring in August 2012 conducted after harvest of barley.

Figure 14. Field CZ05 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 15. Field CZ05 – picture from volunteer monitoring
Field CZ05 at time of first volunteer monitoring in June 2012.

Figure 16. Field CZ05 – picture from volunteer monitoring
Field CZ05 at time of second volunteer monitoring in August 2012.

Figure 17. Field CZ06 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 18. Field CZ06 – picture from volunteer monitoring
Field CZ06 and field surroundings at time of first volunteer monitoring in June 2012.

Figure 19. Field CZ06 – picture from volunteer monitoring
Field CZ06 at time of second volunteer monitoring in August 2012 conducted after harvest of oilseed rape.

Figure 20. Field CZ07 – paths for monitoring
The yellow line indicates a length of 0.1 km; red lines represent the outer border of the area planted with Amflora in 2010; blue and green lines indicate the paths taken through the field to select the observation plots in June and August 2012 respectively.

Figure 21. Field CZ07 – picture from volunteer monitoring
Field CZ07 and field surroundings at time of first volunteer monitoring in June 2012.

Figure 22. Field CZ07 – picture from volunteer monitoring
Field CZ07 at time of second volunteer monitoring in August 2012 conducted after harvest of oilseed rape.