ANNEX 10

STABILITY OF THE AMYLOPECTIN TRAIT IN TUBERS OF AMLFORA STARCH POTATOES GROWN IN 2010

STABILITY OF THE AMYLOPECTIN TRAIT IN TUBERS OF AMFLORA STARCH POTATOES GROWN IN 2010

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ABBREVIATIONS AND DEFINITIONS

CFR	Code of Federal Regulations (USA)
CZ	Czech Republic
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
gbss	Granule bound starch synthase gene

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SUMMARY

The potato variety Amflora (event EH92-527-1) 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 production at locations in the Czech Republic in 2010.

Amflora tubers are characterized by a greater than 98% amylopectin content in the tuber starch fraction due to the reduction of amylose synthesis. As part of the Amflora post-market environmental monitoring plan (EU Register, 2010), the purpose of this study was to confirm that Amflora starch potato tubers grown at field locations in the Czech Republic in 2010 maintain the intended amylopectin trait. Tubers were sampled after harvest from seven locations in the Czech Republic. A total of 28 pooled Amflora samples were analysed by extracting and staining the starch grains with Lugol's iodine solution. An additional eight pooled samples from the conventional variety Bonanza serving as control were also analysed. Lugol's iodine solution stains starch grains from a conventional potato tuber such as Bonanza dark blue due to the presence of amylose. In contrast, starch grains from Amflora tubers are not stained and appear reddish brown due to the near complete absence of amylose. Analysis of all Amflora tuber samples clearly showed the stability of the amylopectin phenotype, thereby confirming the results presented in the Amflora notification C/SE/96/3501 according to Directive 2001/18/EC and verifying the assumption made in the environmental risk assessment.

INTRODUCTION

The potato line EH92-527-1 (OECD Unique Identifier BPS-25271-9) has been genetically modified for increased amylopectin content in the tuber starch. The mother starch potato variety Prevalent was transformed with a construct containing a gene fragment encoding granule bound

starch synthase (*gbss*) from potato in reverse (antisense) orientation under the control of the potato *gbss* promoter. A kanamycin resistance gene from *Escherichia coli* under the control of the nopaline synthase promoter from *Agrobacterium tumefaciens* allowed selection of the transformant in tissue culture. The potato line EH92-527-1 with the variety name Amflora was approved for commercial cultivation in the European Union in March 2010 and was cultivated for starch production in the Czech Republic in 2010.

Tubers of EH92-527-1 potato contain reduced levels of amylose in the starch fraction, concomitant with an increase in amylopectin, the branched-chain starch component, to more than 98% of the total starch content (EFSA, 2006). The purpose of this study was to confirm that Amflora starch tubers grown at field locations in the Czech Republic in 2010 maintain the amylopectin trait. As outlined in the post-market environmental monitoring plan for Amflora (EU Register, 2010), tubers were sampled and pooled after harvest from seven locations in the Czech Republic in 2010, starch was extracted and analysed.

MATERIALS AND METHODS

Source of Plant Material. Amflora potatoes were cultivated for starch production at seven field locations in the Czech Republic in 2010 (Table 1). The tuber sampling followed the outline provided in the post-market environmental monitoring plan for EH92-527-1 potato (EU Register, 2010), which calls for sampling after harvest at the starch potato cultivation sites in four replicates. At each of the seven field location, four pooled tuber samples were collected, each consisting of 10 individual tubers. A total of 28 Amflora pooled tuber samples were prepared in this way. In addition, four pooled tuber samples, each consisting of 10 individual tubers, were taken from the conventional potato variety Bonanza grown in both Kristianstad in Sweden and Baalberge in Germany. These 8 pooled samples served as the control samples for the microscopic analysis of stained starch grains. Starch isolated from the potato variety Prevalent served as the control for the numerical analysis of starch grain staining (Table 1), and was added to an Amflora starch sample (Figure 1, bottom right panel) as a control to show the clear contrast between starch grains containing amylose and Amflora grains without amylose after staining.

Starch Extraction and Staining. Tubers were washed and peeled. A small slice, containing about 1 g of tissue, was taken from each of the 10 tubers in a pooled sample and combined to make a single sample. Pooled samples of tissues containing one slice from 10 individual tubers were crushed together in a mesh bag (LINARIS GmbH, Wertheim-Bettingen, Germany). This treatment produced approximately 2 ml tuber juice, which was transferred into a 2 ml Eppendorf tube. 50 µl of tuber juice was diluted with 50 µl deionised water before 100 µl of 0.3% Lugol`s iodine solution (Kuipers *et al.*, 1994) was added.

Control Material. Potatoes of the conventional variety Bonanza were used as a positive control, as all starch granules from this variety contain amylose and consequently stain blue. Additional controls were prepared by mixing 10 µl of tuber juice from the conventional variety Prevalent with 105 µl of the Amflora tuber juice samples, producing a solution with an amylopectin content of about 96%. These controls show that grains containing amylose could be detected when mixed with amylopectin grains from Amflora tubers.

Detection Level Determination. In order to determine the detection level for amylose in starch extracted by this method, starch extract from a conventional potato tuber containing about 80% amylopectin and 20% amylose was mixed with a starch extract from Amflora tubers, containing > 98% amylopectin (EFSA, 2006). These tuber juices were mixed in different ratios to achieve concentrations of amylopectin between 80% and 98%. Starch of tuber juice mixtures were stained with Lugol`s iodine solution and evaluated under the microscope. A clear gradient can be observed in the dilution series, with the number of deep blue stained starch granules decreasing as the percentage of amylopectin grains increases (Appendix Figure A).

Evaluation of Stained Starch Granules. Stained starch granule solutions were spotted on a microscope slide and observed with an E600 microscope (Nikon Eclipse E600, Germany) at 200x magnification. Each sample was analyzed by observing 3 randomly selected sets of 100 starch grains and recording the dark blue stained grains containing amylose. Each set of 100 grains was located in a different region of the slide.

RESULTS AND DISCUSSION

No dark blue staining granules were visible after staining Amflora tubers harvested from seven different locations in the Czech Republic in 2010, which is consistent with the amylopectin trait (Figure 1). In contrast, all starch granules in samples derived from the conventional potato variety Bonanza showed deep blue stained granules indicating the presence of amylose in each starch granule (Figure 2). Although grains with dark appearance can be seen in the Amflora samples, these grains are only opaque, and they can be clearly differentiated from the dark blue staining amylose grains as can be seen in the panel showing Prevalent granules mixed with Amflora granules (Figure 1).

The analysis of 28 independent samples from Amflora did not detect any dark blue staining granules, meaning that Amflora contains amylose at such a low level that it cannot be detected by this method.

CONCLUSIONS

Whereas starch grains isolated from the conventional potato variety Bonanza are stained dark blue by Lugol's solution due to the presence of amylose, starch granules from Amflora potato tubers are not stained and appear reddish brown due to the near complete absence of amylose. Analysis of 28 independent Amflora tuber samples from field locations in the Czech Republic in 2010 clearly showed the stability of the amylopectin phenotype, thereby confirming the results presented in the Amflora notification C/SE/96/3501 according to Directive 2001/18/EC and verifying the assumption made in the environmental risk assessment.

REFERENCES

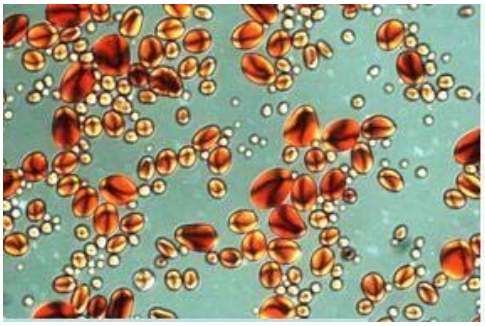
EU Register (2010) Post-market monitoring plan for Notification C/SE//96/3501. Available at: http://ec.europa.eu/food/dyna/gm_register/monitoringplan_eh92-527-1.pdf

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 under:http://www.efsa.europa.eu/de/efsajournal/pub/324.htm

Kuipers, A.G.J., Jacobsen, E., and Visser, R.G.F. (1994) Formation and Deposition of Amylose in the Potato Tuber Starch Granule Are Affected by the Reduction of Granule-Bound Starch Synthase Gene Expression. The Plant Cell 6:43-52.

Figure 1. Light Microscopy Pictures of Stained Starch Grains Derived from Amflora Tubers Grown in the Czech Republic in 2010

Only 1 of 4 replicates from each location is shown. Control panel at bottom right.



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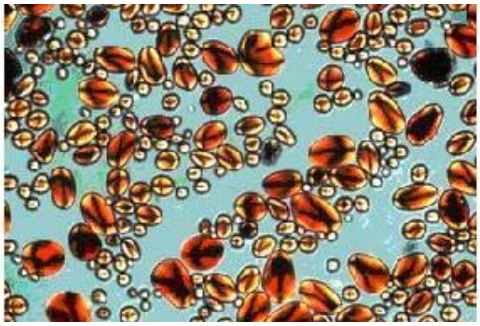

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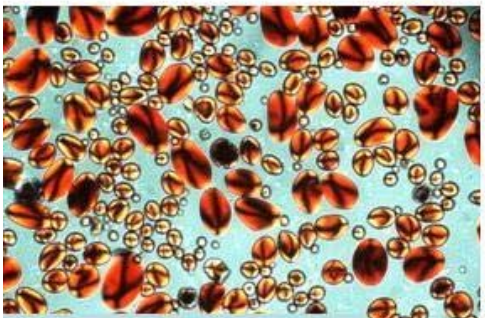

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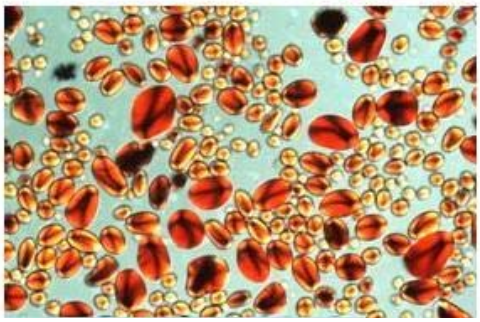

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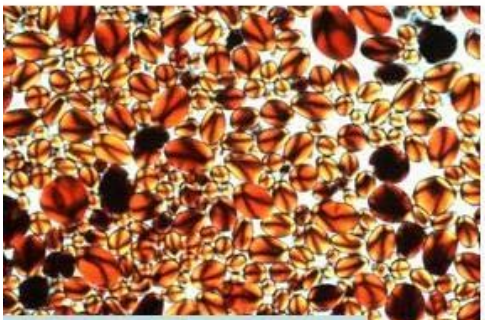

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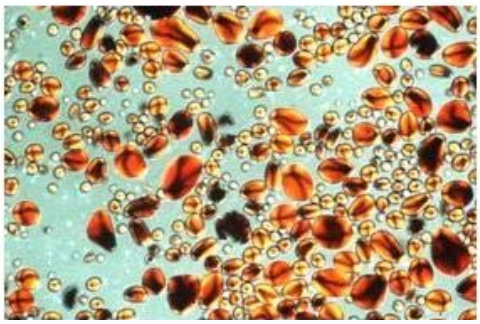

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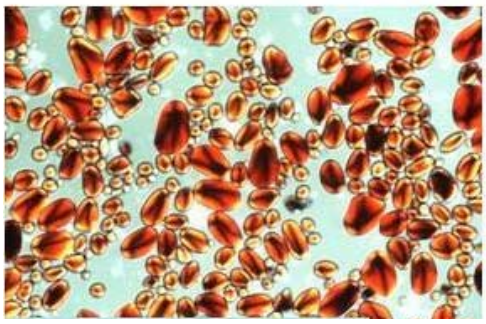

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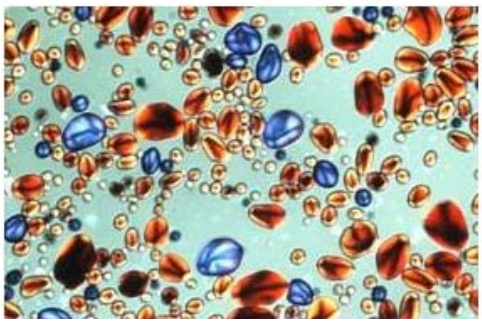
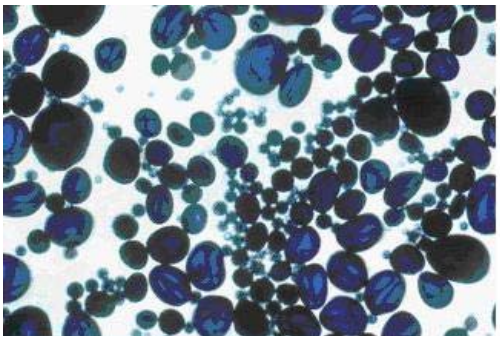


Control: Amflora Mixed with Prevalent

Figure 2. Light Microscopy Pictures of Stained Starch Grains Derived from Conventional Bonanza Tubers Grown in Sweden and Germany in 2010



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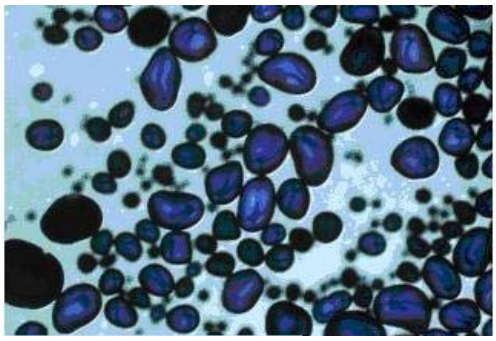

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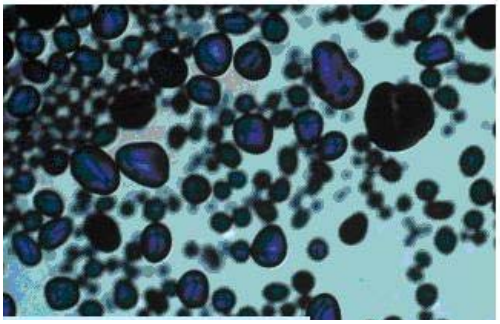

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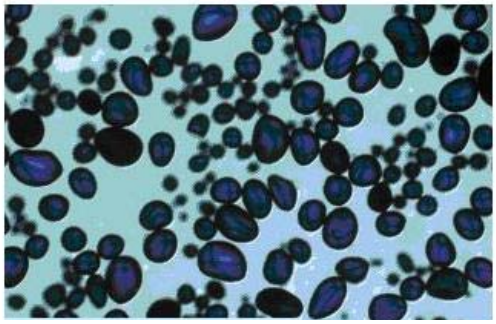

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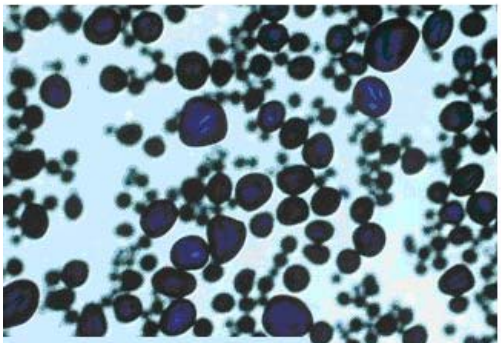

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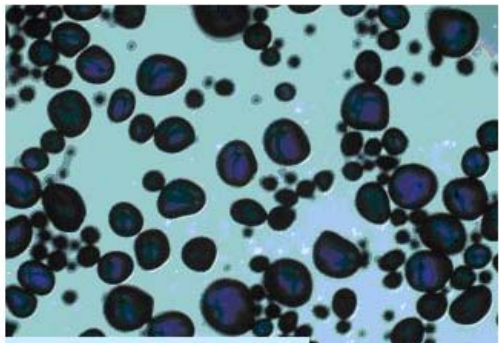
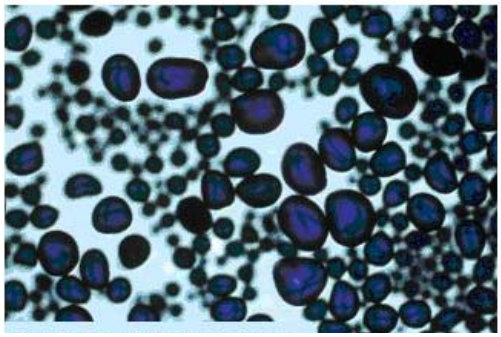
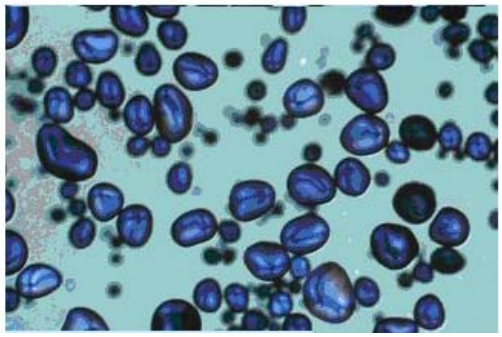


Replicate 3 Kristianstad (SE)



Replicate 4 Baalberge (DE)



Replicate 4 Kristianstad (SE)

Table 1. Numerical Analysis of Amylose Grains in Pooled Tuber Samples Harvested in the Czech Republic in 2010

Potato Variety	Location (Field Identifier)	Rep	Dark Blue Grains Containing Amylose		
Amflora	**CZ01** (630-1120-5502)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ02** (630-1120-4601/1)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ03** (620-1110-7703/1)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ04** (620-1110-7705/6)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ05** (620-1110-7705/2)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ06** (650-1110-0402/11)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100
Amflora	**CZ07** (650-1110-0402/1)	1	0/100	0/100	0/100
		2	0/100	0/100	0/100
		3	0/100	0/100	0/100
		4	0/100	0/100	0/100

Rep = Replicate; CZ = Czech Republic

APPENDIX

Figure A. Light Microscopy Pictures of Stained Starch Grains in Tuber Juice Mixtures Containing an Increasing Amylopectin to Amylose Ratio