Environmental monitoring of GMOs *via* pollen traps

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Aim of the study

Establish a robust approach for molecular analysis of aerosol samples for the presence of genetically modified (GM) pollen

Zea mays pollen as a model

Co-existence & Environmental monitoring

EU legal Framework

Co-existence : co-cultivation of conventional, organic and genetically modified crops

But: Compliance with the organic farming requirements invokes **absence of genetically modified materials** in the products labelled ‘organic’, ‘bio’,…

Thus: in organic farming **zero-tolerance** to GMOs at all stages, starting with cultivation

HOW TO MANAGE?

EU legal Framework

Organisation of EU agriculture in a co-existence (Commission Recommendation of 13 July 2010)

1. Physical and Temporal separation of sources of outcrossing
2. GMO-free cultivation areas

Establishment:
- contract-based cultivation
- isolation distances
- traceability (paper track)

Control:
- seed level
- during cultivation (pollen monitoring)
- post-harvest (grain testing)

Environmental monitoring of GMOs via pollen traps: steps

1. Outdoor exposure of sampling devices

   →Volumetric sampler VPPS 2010 (Lanzoni)

   →Technical pollen sampler PMF/Sigma2 (TIEM)

2. Extraction of pollen grains from the entrapping surface (tape, filters)

3. DNA purification from pollen grains

4. Molecular analysis (Real-Time PCR methods, Pre-spotted plates)

Pollen capture: Volumetric sampler

- ✓ Widely used for sampling the bioaerosol composition of the atmosphere
- ✓ Official sampling method for identification and quantification of airborne pollen from allergenic species
- ✓ Airborne pollen and particles in the range 1-10 µm diameter impact onto an adhesive coated transparent plastic tape (**active sampler**, it requires power supply). Exposure time 1-7 days.
- ✓ The tape is examined microscopically to determine the atmospheric pollen concentration→pollen count: grains of pollen per species per cubic meter over a 24 hour period (Italian Standard UNI 11108:2004)
- ✓ For GMO analysis plastic tape substituted by silicon tape

Hirst type volumetric sampler (Hirst, 1952; Gregory, 1973)

Pollen capture: Technical pollen sampler

(Pollen Mass Filter, PMF)

- ✓ **Specifically conceived for GM pollen monitoring** (long time exposure)
- ✓ It does not require power supply (**passive sampler**)
- ✓ Airborne pollens attach and adhere on filter discs providing low aerodynamic resistance to ambient air passing through the filter
- ✓ The rainwater is collected in a collection flask
- ✓ A protocol has been developed to extract pollen grains from filters leading to pollen samples suitable for DNA extraction and PCR (Hofmann et al., 2008. 1st global conference on GMO analysis)

Technical pollen sampler PMF/SIGMA-2 (VDI guideline, 2007)

Manual maize pollen collection

Drying at 25-30°C on fine flax cloth

Shaking in paper bags

Tassel

Zea mays

Sieving

Pure pollen grains

Storage for 1 year at -20°C

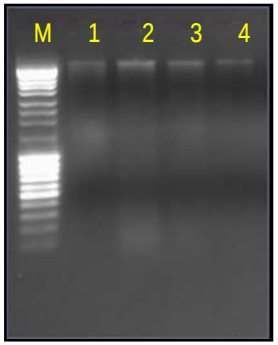
CRA-MAC: Research Unit for maize breeding, Bergamo, Italy

CSIC-IRTA: Consejo Superior de Investigaciones Científicas, Girona, Spain

DNA purification from maize pollen

Optimised protocol:

- ✓ **Mechanical pollen disruption with glass beads (1mm, Sigma)**
- ✓ **CTAB based method with overnight**
- ✓ **lysis RNAse treatment**



0.8% w/v agarose gel electrophoresis. M: 5 µL Mass Ruler (80 bp-10 Kb, Fermentas); 1: 40ng MON863 DNA; 2, 3, 4: 2µl maize pollen DNA purified from 100, 50 and 25mg of pollen respectively.

Yield: 1.9 (±0.21) µg per 100 mg of maize pollen

Recovery: 47 (±5) %

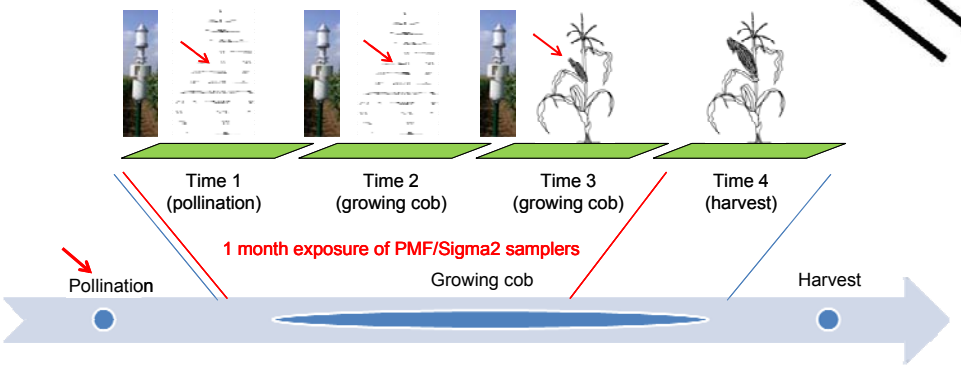
Recovery of pollen grains from silicon tape and PMF filters

PCR-grade pollen DNA could be recovered from both pollen samplers and correctly quantified

2010 field activities

Co-existence field trial in Gent, Belgium (12.5 hectare field) → for pollen capture with PMF/Sigma2 in field conditions (in collaboration with Marc De Loose, ILVO Ghent, Belgium)

In field testing in Belgium



In collaboration with Marc De Loose (ILVO)

Analyses of in-field captured pollen on PMF

Sample	Sample info	# pollens analyzed	PCR (hmg)	PCR (MON810)	Yield %	GM % HGE	DNA quant (Picogreen)
Positive Control	2-3% Mon810. Germany 2008	$40*10^3$	+	+	14	3.56%	800 pg/µl
Positive Control	MON810 positive Germany 2006	$10*10^3$	+	+	4	52.66%	200 pg/µl
Positive Control	MON810 positive Germany 2008	$20*10^3$	+	+	6	8.53%	200 pg/µl

✓ PCR Results are in line with the positions of the traps with respect to the GM source

✓ Inhibition of PCR reaction was observed

PMF5	Ghent 2010	$17*10^3$	+	+		nq	Out of scale
PMF6	Ghent 2010	$12.8*10^3$	+	+		nq	Out of scale
PMF7	Ghent 2010	$7.4*10^3$	-	-			Out of scale
PMF8	Ghent 2010	$30.4*10^3$	+	-			Out of scale

nq: not present in quantifiable amount

(**#** indicates the number of pollen used for DNA extraction as counted by microscopic count; ***** PMF4 was further purified through microsieving)

MON 810 Spain

Collection of MON 810 pollen in collaboration with Maria Pla, CSIC Barcelona, Spain

MON 810 cultivation in Girona (Spain)

In house pollen production

Event	Number of plants
MON863xNK603	6
MON88017	4
MON863	4
MON863xMON810	6
MON 863xMON810xNK603	4
MON88017xMON810	4
NK603xMON810	4
MON810	2
NK603	2

List of plants in the growth chamber

Growing conditions: 25°C, 75% humidity, 16 hs of daylight – 8 hs of darkness, light brightness 10000-15000 lux

Identification of GM pollen samples using the Real-Time PCR-based Ready-to-Use Multi-Target Analytical System

	1	2	3	4	5	6	7	8	9	10	11	12
A	HMG Maize Ref	HMG Maize Ref	Bt11 Maize	NK603 Maize	GA21 Maize	MON863 Maize	DAS1507 Maize	T25 Maize	DAS59122 Maize	MON810 Maize	MIR604 Maize	MON88017 Maize
B	LY038 Maize	3272 Maize	MON89034 Maize	98140 Maize	Lectin Soybean Ref	Lectin Soybean Ref	A2704-12 Soybean	40-3-2 Soybean	MON89788 Soybean	DP-356043 Soybean	DP-305423 Soybean	A5547-127 Soybean
C	HMG Maize Ref	HMG Maize Ref	Bt11 Maize	NK603 Maize	GA21 Maize	MON863 Maize	DAS1507 Maize	T25 Maize	DAS59122 Maize	MON810 Maize	MIR604 Maize	MON88017 Maize
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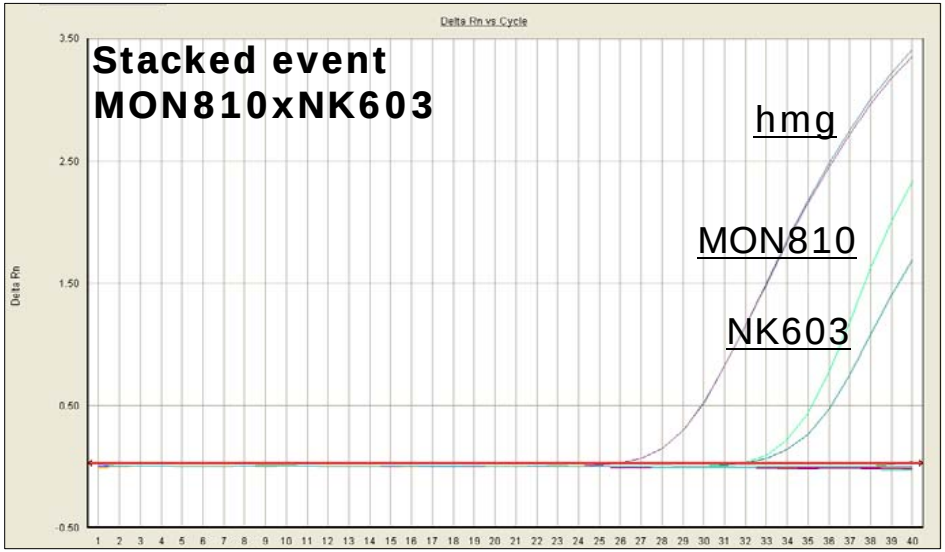
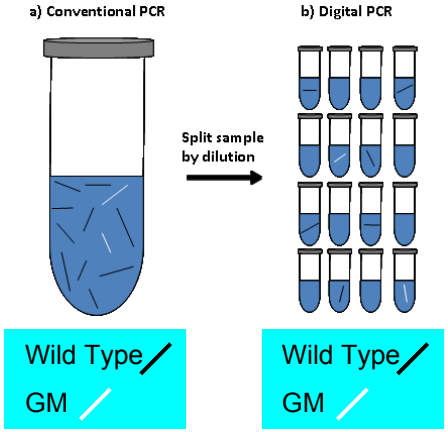
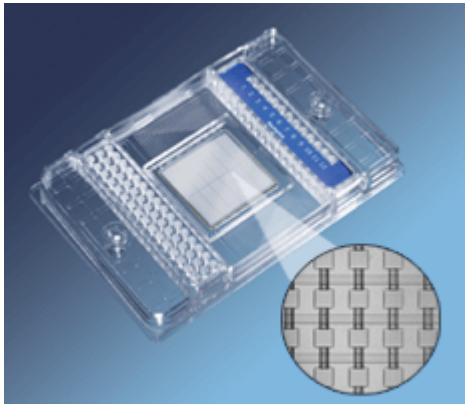


Sample	Origin WT	Origin GM	hmg	MON810	NK603
5% MON810	Spain	Spain	27.03	33.35	nd
5% NK603	Spain	In house	27.14	nd	33.11
5% MON810xNK603	Spain	In house	26.61	32.28	32.57
5% MON810-Silicon	Spain	Spain	28.95	34.79	nd

Digital PCR

Digital PCR works by partitioning single DNA samples into hundreds individual PCR reactions at a concentration at which only a fraction of the reactions contains one or more target molecules. These reactions will produce PCR products and can therefore be detected. By counting the number of positive reactions the number of target molecules in the sample can be accurately estimated [NucleicAcidsRes.25,1999–2004,1997; Proc.NatlAcad.Sci.USA.96,9236–41,1999].



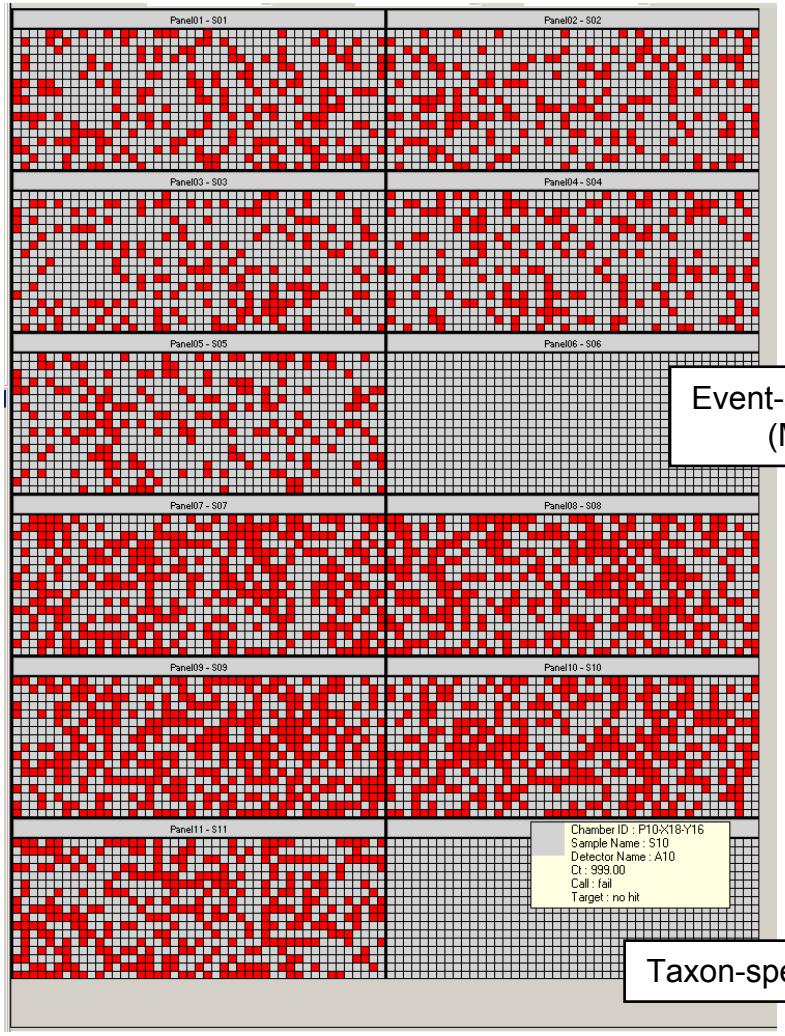


Fluidigm BioMark digital chip

Real-time PCR instrument by Fluidigm

Microfluidic channels split each sample into 765 reaction chambers (inset) prior to standard thermal cycling and real-time PCR analysis

Digital PCR in GMO analysis



Example of an analysis by digital PCR: Positive hits in the partitions

! Calculation of the GM% is made independent of any calibrator

$$\text{GM\% in haploid genome copies} = \frac{\text{Number of positive partitions for the event-specific assay}}{\text{Number of positive partitions for the taxon-specific assay}} * 100$$

Results from dPCR on MON810 leaf tissue

Assay	Range Min	Range Max	Count	nVolume	Est. Targets	Est.T-95L	Est.T-95U
MON810	20	35	208	1	243	209	276
MON810	20	35	180	1	205	175	235
MON810	20	35	169	1	191	162	219
MON810	20	35	176	1	200	170	229
MON810	20	35	180	1	205	175	235
MON810	20	35	0	1	0	0	0
hmg	20	35	331	1	434	387	481
hmg	20	35	336	1	443	395	491
hmg	20	35	363	1	493	441	545
hmg	20	35	331	1	434	387	481
hmg	20	35	312	1	401	356	446
hmg	20	35	0	1	0	0	0
%GM					**47**	**45**	**49**

Future analyses in pollen monitoring activity

1. New field activities (Brazil*, Mexico,…)
2. Environmental PCR inhibitors
3. Molecular detection tools (digital PCR, RT-PCR…)

*Collaboration with EMBRAPA (Brazil) ongoing (MoU)

On-field pollen entrapment experimentation in commercial GM maize fields

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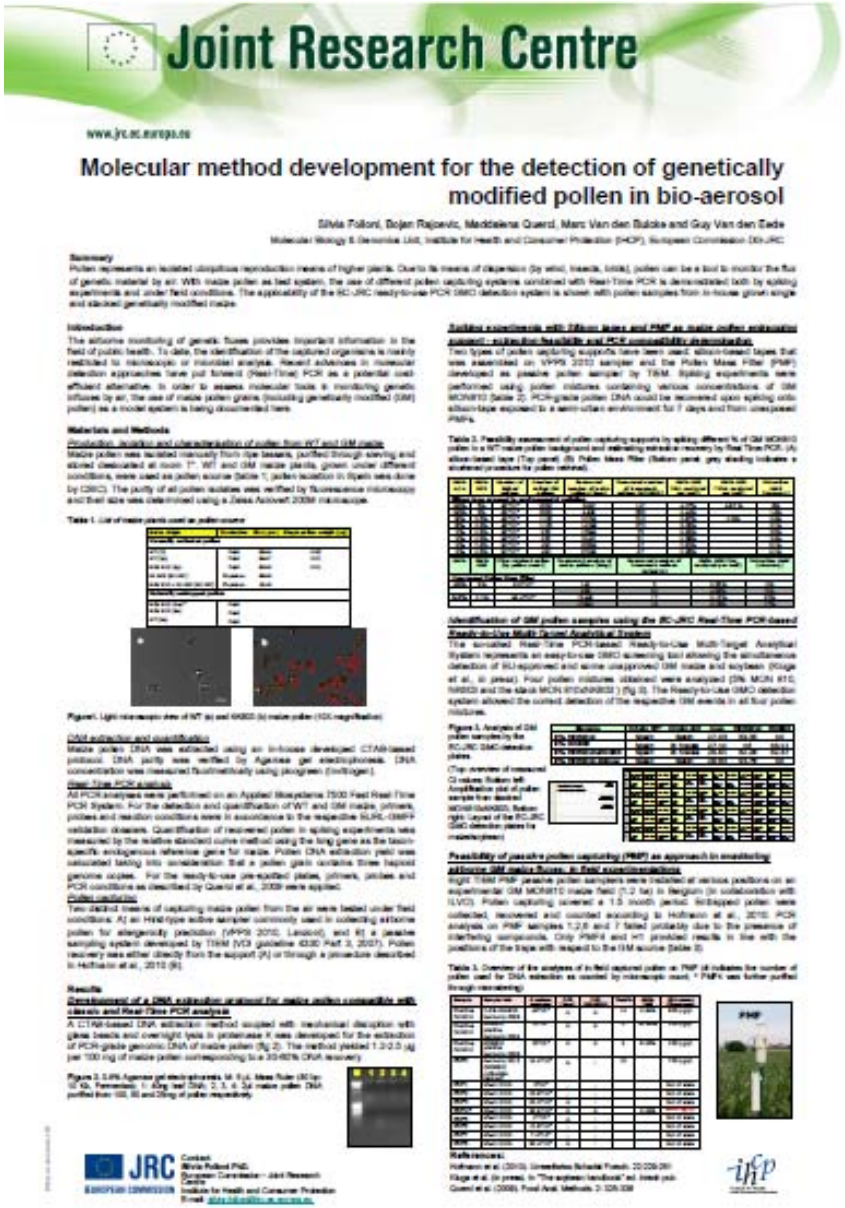
Joint Research Centre

www.jrc.ec.europa.eu

Molecular method development for the detection of genetically modified pollen in bio-aerosol

Silvia Folloni, Bojan Rajcevic, Maddalena Querci, Marc Van den Bulcke and Guy Van den Eede

Molecular Biology & Genomics Unit, Institute for Health and Consumer Protection (IHCP), European Commission (DG JRC)

Summary

Introduction

Materials and Methods

Results

Discussion

References

Thank you!

Gracias!