Training session: comet assay on plants

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Quick introduction

• MSc in Plant physiology and molecular biology (Univ of Toulouse)

• PhD in Ecotoxicology: molecular mechanism of lead toxicity in plants (Univ. of Toulouse)

• Since 2008: Ass. Prof. in Environmental Science – Ecotoxicology (ISA Lille – Univ of Lille):
  ✓ Phytoremediation of contaminated sites
  ✓ Phytomanagement of contaminated sites

  → comet assay is a tool to select suitable plant

  ✓ Ecotoxicology: impact of pollutants on plants
    • Ecotoxicology: in lab
    • Biomonitoring on the field

  → comet assay is a tool to assess negative impact on plants
First works

- 2007: start of the studies on Comet assay on *Vicia faba*

Gichner’s nucleus extraction
First works

• 2007: start of the studies on Comet assay on *Vicia faba*

Gichner’s nucleus extraction
Filtration 20 µM
Unwinding
15 min

Migration
10 min

Lysis
1H

Mix LMPA
1:1

Filtration
20 µM

70 µL
Scoring and data analysis

• Recommendation of Comet assay workgroup (Hartmann et al., 2003): 150 nuclei scored per samples:
  - 50 nuclei / slide;
  - 3 slides / sample;
  - at least 5 samples (plants) / treatment

• Visual scoring (calibration) and Komet software (experimentation)
Lead-induced DNA damage in *Vicia faba* root cells: Potential involvement of oxidative stress

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\(^d\) Université Lille Nord de France, F-59000 Lille, France
Comet assay on plants: limits and challenges

- Extraction yield: 20-50 nuclei / slide

  Scoring: 150 nuclei / sample
  → 40-50 nuclei / slide; 3-4 slides
Comet assay on plants: limits and challenges

- **Extraction yield:** 20-50 nuclei / slide
- **Time consuming:**
  - Nucleus extraction step: 20 samples /day (+ controls)
  - Scoring step: 10 samples /day

→ not suitable for biomonitoring studies (hundreds/thousands of samples)
Comet assay on plants: limits and challenges

• Extraction yield: 20-50 nuclei / slide

• Time consuming:

• Lack of standardization – need to highlight the protocol keypoints
  - Most of researchers use a protocol derivated from Gichner’s ones
  - Lysis/No lysis
  - Filtration/no filtration
  - Number of scored nuclei
  - …
Comet assay on plants: limits and challenges

- Extraction yield: 20-50 nuclei / slide
- Time consuming:
- Lack of standardization – need to highlight the protocol keypoints
- Lack of repeatability/reliability: Operator-related variation

![Graph showing % tail DNA vs Experimentation number (control plants)]
Comet assay on plants: limits and challenges

- Extraction yield: 20-50 nuclei / slide
- Time consuming:
- Lack of standardization – need to highlight the protocol keypoints
- Lack of repeatability/reliability: Operator-related variation

Several labs gave up!

Main reasons:
- No standardized protocol
- Lack of calibration
Filtration
20 µM

Mix LMPA
1:1

Unwinding
15 min

Lysis
1H

Migration
10 min

70 µL
**Filtration step**

*Not realized by Gichner*

<table>
<thead>
<tr>
<th>Filtration 20 µM</th>
<th>Nb nucleus /slide</th>
<th>DNA damage (% in the tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>155.3 ± 12.3</td>
<td>5.9 ± 0.9</td>
</tr>
<tr>
<td>No</td>
<td>67.3 ± 27.3*</td>
<td>15.2 ± 3.1*</td>
</tr>
</tbody>
</table>

**Significant gain:**
- nucleus number / slide
- nucleus quality
- time

**However: More fragments - Very important background**
→ Difficulties for analysis with Comet analysis softwares

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Filtration
20 µM
Mix LMPA
1:1
Filtration
20 µM
Mix LMPA
1:1
Filtration
20 µM
Mix LMPA
1:1
Unwinding
15 min
Lysis
1H
Migration
10 min
70 µL
Nucleus extraction step

The most sensitive step in comet assay on plant

Slicing vs chopping?

Slicing: low extraction yield not compatible
- with robust statistical analysis
- with medium (12 gels) and high throughput comet assay (96 gels)

<table>
<thead>
<tr>
<th>Nucleus extraction</th>
<th>Filtration (20 µm)</th>
<th>Number of nuclei per slide</th>
<th>DNA damage (% DNA in the tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slicing</td>
<td>No</td>
<td>31.3 ± 11.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>16.1 ± 3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.1 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chopping 15s</td>
<td>No</td>
<td>99.4 ± 12.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>45.9 ± 21.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.1 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chopping 30s</td>
<td>No</td>
<td>155.3 ± 12.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.9 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>67.3 ± 27.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>15.2 ± 3.1&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chopping 45s</td>
<td>No</td>
<td>237.6 ± 23.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.5 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>88.0 ± 34.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>24.3 ± 6.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chopping 60s</td>
<td>No</td>
<td>383.1 ± 32.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.7 ± 5.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>153.1 ± 23.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>56.9 ± 7.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
The most sensitive step in comet assay on plant

Slicing vs chopping?

Solve the equation:

\[ x \text{ min of chopping} = \text{sufficient nb of nucleus + nucleus the less degraded as possible} \]

\[ \uparrow \text{chopping time} = \uparrow \text{nb nucleus} \uparrow \text{background} \uparrow \text{DNA damages} \]

\[ \downarrow \text{chopping time} = \downarrow \text{nb nucleus} \downarrow \text{background} \downarrow \text{DNA damages} \]
Unwinding
15 min

Migration
10 min

Mix LMPA
1:1

Lysis
1H

What's for?

70 µL
Lysis step

Not performed by Gichner and apparently useless
But still 30-40% of researchers still include a lysis step

<table>
<thead>
<tr>
<th>EMS exposure (mM)</th>
<th>Lysis</th>
<th>DNA damage (% DNA in the tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td>93.6 ± 3.3</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>89.1 ± 2.2</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>75.7 ± 2.7</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>74.6 ± 2.4</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>55.3 ± 3.1</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>51.8 ± 3.2</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>35.3 ± 2.8</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>31.3 ± 2.1*</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>14.2 ± 1.1</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>4.7 ± 0.6</td>
</tr>
</tbody>
</table>

Lysis step must be skipped as:
- it is unnecessary, time-consuming,
- does not improve comet assay results and could induce bias in the assay.

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Mix LMPA 1:1
Unwinding 15 min
Migration 10 min
70 µL
Objectives:
- Increase CA sensitivity
- Save time
Dénaturation
15 min

Calibration?

Migration
10 min

Mélange LMPA
1:1

70 µL
Calibration

Irradiation is the best method

Figure 3. Dose-response curves of DNA damages (% DNA in the tail) of nuclei isolated from (A) V. faba roots (open circles) treated with H$_2$O$_2$ in Hoagland’s solution for 1 h; (B) V. faba roots (full circles) and leaves (open square) exposed to EMS in Hoagland’s solution for 24 h. Each value represents mean and associated standard error (n=6).

Easy to set up in hydroponic conditions H$_2$O$_2$ or mutagens (MMS, EMS)
Calibration

Irradiation is the best method

Figure 4. Dose-response curves of DNA damages (% DNA in the tail) of nuclei isolated from leaves of *L. perenne* (full diamonds), *T. repens* (open triangles up) and *M. giganteus* (open circles) grown on control soils and watered with EMS in mixed water (tap: osmosed water, 1:4) for 4 days. Each value represents mean and associated standard error (*n*=6).

Possible to calibrate using soils and mutagens (EMS works fine!)

→ generation of hazardous wastes

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Calibration

Take in consideration leaf position!

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Mélange LMPA 1:1

Dénaturation 15 min

Migration 10 min

Repeatability?

70 µL
Repeatability

50 nuclei / slide; 3 slides / sample; at least 5 samples (plants) / treatment (Hartmann et al., 2003)
Repeatability

50 nuclei / slide; 3 slides / sample; at least 5 samples (plants) / treatment (Hartmann et al., 2003)

- effects of room temperature on Comet assay results

![Graph showing the effects of room temperature on Comet assay results. The x-axis represents temperature in °C, ranging from 0 to 45. The y-axis represents score (ua), ranging from 0 to 450. Different data points are distinguished by color: blue for 20-25°C, pink for >25°C, and yellow for <20°C. The graph includes a red circle highlighting the data points for 20-25°C.](rye-grass)
Repeatability

50 nuclei / slide; 3 slides / sample; at least 5 samples (plants) / treatment (Hartmann et al., 2003)

- effects of room temperature on Comet assay results

![Graph showing the influence of room temperature on DNA damages of nuclei isolated from control and EMS-treated V. faba plants.](30)

**Figure 1.** Influence of room temperature on DNA damages of nuclei isolated from control and EMS-treated V. faba plants. Full diamonds and open triangles up represent roots and leaves of control V. faba, respectively, grown in Hoagland’s solution. Full circles and open squares represent roots and leaves of V. faba, respectively, exposed to 4 mM EMS in Hoagland’s solution for 24h. Each value represents mean and associated standard error (n=6). *Values significantly different from the control (P < 0.05).*

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Repeatability

50 nuclei / slide; 3 slides / sample; at least 5 samples (plants) / treatment (Hartmann et al., 2003)

- effects of room temperature on Comet assay results
- effects of light on Comet assay results

Pourrut et al. Recommendations for increasing alkaline comet assay reliability in plants. Mutagenesis (2015)
Unwinding 15 min

Migration 10 min

Mix LMPA 1:1

70 µL
Medium-throughput: protocol

• Same extraction method: chopping with razor blade
• Some modifications in Gichner’s protocol to increase nucleus density (10 µL drop vs 70 µL in standard protocol)
• 12 gel slide (Shaposhnikov et al., Toxicology Letters 2010)

(from Shaposhnikov et al., 2010)

12 gels: 3 x control + 3 x 3 samples
Medium-throughput: plant treatment

- 8 week Ryegrass (*Lolium perenne*) and White clover (*Trifolium repens*)
- Control plants (watered with water) vs positive control plants (watered with 50 mM EMS water for 4 days)

Standard protocol:
- 5 controls
- 5 EMS

Medium-throughput:
- 5 controls
- 5 EMS
Medium-throughput protocol:

- Is as sensitive as standart protocol
- Reduces intra- and inter-experimentation variations
- Is 3 times faster (no coverslip, 1 slide vs 12 in the electrophoresis tank...)

No difference within 12 gel slides
Medium-throughput protocol:

- Is as sensitive as standard protocol
- Reduces intra- and inter-experimentation variations
- Is 3 times faster (no coverslip, 1 slide vs 12 in the electrophoresis tank...)
- Saves time during scoring (>5 times faster)
Medium-throughput protocol:

- Compatible with automated scoring systems
- Project on going with IMSTAR
- 4h of analyse vs 10 days
Application to polluted soils

- Soils artificially contaminated with EMS (Ethyl Methane sulfonate)

- Dose-depandanant response

\[ T. \text{repens} \quad \text{L. perenne} \]
Evaluation of heavy metal contaminated soils phytotoxicity

Dose-dependant genotoxicity

50% DNA damages $[\text{Pb}] \geq 800 \text{ mg kg}^{-1}$

<table>
<thead>
<tr>
<th>Soil</th>
<th>$[\text{Pb}]$ mg kg$^{-1}$</th>
<th>$[\text{Cd}]$ mg kg$^{-1}$</th>
<th>$[\text{Zn}]$ mg kg$^{-1}$</th>
<th>pH$_{\text{water}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32</td>
<td>0.6</td>
<td>76</td>
<td>6.9</td>
</tr>
<tr>
<td>2B</td>
<td>101</td>
<td>1.9</td>
<td>150</td>
<td>6.6</td>
</tr>
<tr>
<td>T 216</td>
<td>131</td>
<td>6.9</td>
<td>773</td>
<td>7.5</td>
</tr>
<tr>
<td>17B</td>
<td>240</td>
<td>4.9</td>
<td>307</td>
<td>7.5</td>
</tr>
<tr>
<td>28B</td>
<td>451</td>
<td>8.8</td>
<td>587</td>
<td>7.8</td>
</tr>
<tr>
<td>35B</td>
<td>777</td>
<td>14.9</td>
<td>1000</td>
<td>8</td>
</tr>
<tr>
<td>33B</td>
<td>1079</td>
<td>19.2</td>
<td>1371</td>
<td>7.9</td>
</tr>
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White clover ($T. \text{repens}$)

* Values significantly different from the control (P<0.01).
Application to polluted soils

- Evaluation of heavy metal contaminated soils phytotoxicity

<table>
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<tr>
<th>Soil</th>
<th>[Pb] mg kg⁻¹</th>
<th>[Cd] mg kg⁻¹</th>
<th>[Zn] mg kg⁻¹</th>
<th>pH&lt;sub&gt;water&lt;/sub&gt;</th>
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<td>7.9</td>
</tr>
</tbody>
</table>

→ High genotoxicity
80% DNA damages
\([\text{Pb}] \geq 800 \text{ mg kg}^{-1}\)

→ difference of sensitivity

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* Values significantly different from the control (P<0.01).
Application to polluted soils

- Selection of suitable plants for polluted site phytomanagement

→ Early evaluation of plant tolerance to pollutants (good correlation with other biomarkers: Miscanthus >> White clover >> Ryegrass

→ Validation du choix du miscanthus pour le phytomanagement du site Metaleurop
Thank for your attention!

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