The use of comet assay in plant toxicology: recent advances

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The systematic study of genotoxicity in plants induced by contaminants and other stress agents has been hindered to date by the lack of reliable and robust biomarkers. The comet assay is a versatile and sensitive method for the evaluation of DNA damages and DNA repair capacity at single-cell level. Due to its simplicity and sensitivity, and the small number of cells required to obtain robust results, the use of plant comet assay has drastically increased in the last decade. For years its use was restricted to a few model species, e.g., Allium cepa, Nicotiana tabacum, Vicia faba, or Arabidopsis thaliana but this number largely increased in the last years. Plant comet assay has been used to study the genotoxic impact of radiation, chemicals including pesticides, phytocompounds, heavy metals, nanoparticles or contaminated complex matrices. Here we will review the most recent data on the use of this technique as a standard approach for studying the genotoxic effects of different stress conditions on plants. Also, we will discuss the integration of information provided by the comet assay with other DNA-damage indicators, and with cellular responses including oxidative stress, cell division or cell death. Finally, we will focus on putative relations between transcripts related with DNA damage pathways, DNA replication and repair, oxidative stress and cell cycle progression that have been identified in plant cells with comet assays demonstrating DNA damage.

Keywords: plant comet assay, genotoxicity, metal, phytocompounds, radiation, pollutants, nanoparticles, DNA damages biomarkers

Plant Comet Assay: General Considerations

The first reports on the use of comet assay in plants date from the 1990’s (e.g., Cerda et al., 1993; Koppen and Verschaeve, 1996; Navarrete et al., 1997; Koppen and Angelis, 1998). Despite similarities with other eukaryotic systems, namely animal models, the comet assay protocols for plants take into account relevant differences including the presence of a rigid cell wall in plant cells. The localized presences of characteristic meristematic regions (e.g., the concentration of highly dividing cells in the root apex) and the fact that root is usually the organ directly in contact with contaminated soil and water, have also influenced the establishment of plant comet assays in ecoxicological approaches. Technical details concerning plant comet assays in different organs and species have been thoroughly reviewed by Gichner et al. (2009).

For almost a decade, the comet assay remained restricted to some toxicological studies and to a few model species including Allium cepa, Nicotiana tabacum, Vicia faba, and Arabidopsis thaliana (for review, Gichner et al., 2009; Ventura et al., 2013).
Plant comet assay has been applied to an increasing variety of adverse conditions. Some recent reviews on this subject (Gichner et al., 2009; Ventura et al., 2013) revised most relevant advances in plant comet assay up to 5 years ago. Since then an increasing interest for comet assay in plants was shown (136 articles published between 2010 and March 2015 vs. 89 between 1995 and 2009). Therefore, here we will mostly emphasize most relevant advances within the last 5 years, and highlight current applications of this technique in plant (eco) toxicological studies. We will also discuss advances on genetic studies involving DNA damage and repair.

**Basic Principles and Methodologies**

Comet assays traditionally use cell suspensions, which are embedded in agarose on a microscope slide, and exposed to lysis by exposure to detergent and high salt solutions (for review Collins et al., 2008; Azqueta et al., 2009). Lysis allows removing membranes and soluble cell components, leaving a supercoiled DNA nucleoid (Azqueta et al., 2011b). When submitted to electrophoretic conditions, DNA fragments will migrate toward the anode, forming a typical "comet tail." The amount of strand breaks is overall proportional to the amount of DNA in the tail respectively to the DNA remaining in the head (Hovhannisyan, 2010).

However, in plants, the presence of a cell wall causes technical issues for performing the comet assay on plant tissues. To overcome these problems, a simple and efficient mechanical extraction to isolate cell nuclei was developed by Cerda et al. (1993), and then improved by Koppen and Angelis (1998), Navarrete et al. (1997), and Gichner and Plewa (1998). Since then, most of the researchers used directly those protocols or derived versions, such as described in Gichner and Plewa (1998). Recently, Pourrut et al. (2015) identified the key steps of comet assay in plants and proposed an optimized protocol to increase its reliability and its throughput. In the case of plant chopping, particular attention has to be paid to the presence of chloroplasts as they are important sources of free radicals and oxidative damage. For example, the first article on plant comet assay testing chemicals used isolated nuclei of *Vicia faba* root cells (Koppen and Verschaeye, 1996). In cellular assays, plants exposed to suspected genotoxicants are processed for nuclei isolation and analysis, whereas in acellular assays, nuclei from non-stressed plants are isolated and then incubated with the genotoxicants, before comet assay analysis.

The use of protocol variants allows detecting a wide range of DNA damages (see for review Angelis et al., 1999; Collins et al., 2008). Briefly, an alkaline treatment (referred hereafter as A/A) and electrophoresis at pH 13 or higher allows the detection of most single and double DNA strand breaks (SSBs and DSBs) and also alkali-labile sites. When the unwinding and subsequent electrophoresis are performed using a buffer pH~7–8, the comet assay is called "neutral" (N/N). A crucial difference is that at alkaline conditions, apurinic/apyrimidinic sites are more easily subjected to break (for details refer to Azqueta et al., 2011b). Other pH-variants (e.g., A/N) have meanwhile been introduced as alternative comet assays.

Moreover, the information provided by comets may also be increased by exposing the DNA to enzymes recognizing a specific lesion, e.g., formamidopyrimidine DNA glycosylase, Endonuclease III, thereby originating specific breaks. However, despite their strong interest and their early introduction in plant studies (Menke et al., 2000), these enzymes are still not much used in plants.

Comets may then be visualized by microscopy, by using a suitable DNA-binding dye, e.g., fluorescent dyes or silver staining. Data can be analyzed by visual scoring, ranging from 0 to 4 according to the damage class, or using computer-based image analysis (e.g., the software http://casplab.com/) that allows the quantification of several comet parameters, including the tail DNA %, tail length, tail extension moment or Olive tail movement (Azqueta et al., 2011b). Criteria for the best scoring approaches are however debatable (e.g., Azqueta et al., 2011a), but independently of the approach and scoring, it is consensual that this technique allows collecting data suitable for robust statistical analyses.

**Radiation**

Plants are prone to DNA damage upon exposure to radiation from natural or anthropogenic sources. For this reason, the analysis of DNA damage in irradiated plants is a topic of growing interest and sensitive methods for detection of DNA damage have been applied (Table 1).

The effects of light excess on plant DNA using comet assay were firstly investigated by Ojima et al. (2009) on *Raphanus sativus* protoplasts. These authors demonstrated that light excess causes DNA degradations mediated by oxidative stress. In 2010, Nishioka et al. confirmed the role of reactive oxidative species (ROS) in light excess-induced DNA damages in *Ipomoea aquatic* root protoplasts, and correlated DNA damages observed by comet assay with chlorophyll degradation. However, these two studies did not take into consideration the potential role of UV in light-induced DNA damages. In a study designed to investigate UV-A and UV-B effects, Jiang et al. (2007) performed comet to detect specific DNA lesions as well as pyrimidine dimers formation (using T4 endonuclease V) in irradiated *Spirodela polyrhiza* protoplasts. These results were confirmed later in *Arabidopsis thaliana* root tip cells (Jiang et al., 2009, 2011). Jiang et al. (2011) also demonstrated that UV-B-induced DNA damage results in the delay of G1-to-S transition of plant cell cycle. However, by using a neutral comet assay (N/N variant), Roy et al. (2011) showed that UV-B-induced lesions were reversible, particularly in *A. thaliana* wild-type (Col-0), compared to DNA polymerase λ. UV-B sensitive mutants. UV-C was also shown to induce both SSBs and DSBs in *Arabidopsis plumbaginifolia* protoplasts (Abas et al., 2007). These authors also highlighted the usefulness of the comet assay as an analytical tool for the analysis of repair kinetics in protoplasts. These results were confirmed by Bilichak et al. (2014) on *A. thaliana* protoplasts.

Besides natural exposure to radiation, plants are also irradiated for industrial purposes. For example, gamma (γ)-rays are used to increase seed vigor and/or enhance plant tolerance to environmental stresses. Navarrete et al. (1997) pioneered the...
| Stress   | Species             | Tissue               | Maximum dose | Nuclei         | Comet type   | Electrophoresis | Analysis | References                |
|----------|---------------------|----------------------|--------------|----------------|--------------|----------------|----------|---------------------------|
| Radiation |                     |                      |              |                |              |                |          |                           |
| Light    | A. thaliana         | Leaves               | 1300 μmol m⁻² s⁻¹ | Galbraith      | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | QTM      | Zeng et al., 2010         |
|          | L. aquatica         | Roots                | 22 W m⁻²      | PBS            | A/A pH > 13 | 25 V, 300 mA 10 min, 4°C | N         | Nishikawa et al., 2010    |
|          | R. sativus          | Cell suspension      | 430 W m⁻²     | PBS            | A/A pH > 13 | 25 V, 300 mA 10 min, 4°C | N         | Ojima et al., 2009        |
| UV       | A. thaliana         | Leaves               | 0.5 W m⁻²     | UV-B, UV-A     | Galbraith,T4endoV | A/A pH > 13 | 0.72 V/cm, 300 mA, 15 min, 4°C | QTM      | Jiang et al., 2009        |
|          |                     |                      |              |                |              |                |          |                           |
|          |                     |                      |              | PBS            | A/A pH > 13 | 1 V/cm, 12 mA 5 min | %TD      | Roy et al., 2011          |
|          |                     |                      |              |                |              |                |          |                           |
|          |                     |                      |              | PBS-EDTA       | NaN pH 8     | 0.7 V/cm, 300 mA, 20 min | %TD      | Abas et al., 2007         |
|          | N. plumbaginifolia  | Cell suspension      | 236 J m⁻²     | PBS-EDTA       | NaN pH 8     | 2 V/cm, 10 mA, 2 min | %TD      | Abas et al., 2007         |
|          | S. polyrhiza        | Protoplasts          | 0.5 W m⁻²     | UV-B, UV-A     | Tris, T4 endoV | A/A pH > 13 | 0.72 V/cm, 300 mA, 15 min, 4°C | QTM      | Jiang et al., 2007        |
|          | (foodstuffs)        | Various              | 5 kGy γ-ray   | PBS, Mg free   | NaN pH 8.4   | 2 V/cm, 2 min | VS       | Cerda et al., 1997        |
|          | (foodstuffs)        | Various              | 10 kGy γ-ray  | PBS-EDTA       | NaN pH 8.4   | 0.66-0.83 V/cm, 300 mA, 5-40 min | %TD      | Verbeek et al., 2008      |
|          | (foodstuffs)        | Seeds                | 1 kGy γ-ray   | PBS            | NaN pH 8.4   | 2 V/cm, 2 min | TL       | Navarete et al., 1997     |
|          | A. cepa             | Roots                | 4 Gy γ-ray    | Sörensen(mod)  | A/A pH > 13  | 0.65 V/cm, 230 mA, 20 min, 10°C | TL/HD    | Koppen and Cerda, 1997    |
|          |                     |                      |              |                |              |                |          |                           |
|          | A. thaliana         | Roots, leaves        | 3 Gy γ-ray    | PBS            | NaN pH 8.4   | 2 V/cm, 10 mA, 2 min, 4°C | VS       | Vandenhove et al., 2010   |
|          |                     |                      |              | PBS-EDTA       | A/A pH 8.4   | 0.7 V/cm, 4°C | %TD      | Moreno-Promero et al., 2012 |
|          | H. vulgare          | Roots                | 110 G y-ray   | Sörensen(mod)  | NaN pH 8     | 10 V/cm, 120 mA, 40 min, 4°C | %TD      | Stolov et al., 2013       |
|          |                     |                      |              |                |              |                |          |                           |
|          | M. truncatula       | Cell suspension      | 50 G y-ray    | Tris           | A/A pH > 13  | 0.65 V/cm, 230 mA, 20 min, 4°C | QTM      | Saghizadeh et al., 2008   |
|          |                     |                      |              |                |              |                |          |                           |
|          | N. tabacum          | Roots, leaves        | 40 G y-ray    | Sörensen(mod)  | A/A pH > 13  | 0.72 V/cm, 300 mA, 20 min, 4°C | QTM      | Gichner et al., 2000      |
|          | O. sativa           | Shoots               | 200 G y-ray   | PBS-EDTA       | A/A pH > 13  | 0.72 V/cm, 20 min, 4°C | VS       | Macovei and Tuteja, 2013  |
|          | P. x hybrida        | Roots                | 200 G y-ray   | PBS-EDTA       | A/A pH > 13  | 1 V/cm, 8 min | VS       | Macovei et al., 2014      |
|          |                     |                      |              |                |              |                |          |                           |
|          | P. nigra            | Cell suspension      | 100 G y-ray   | Sörensen(mod)  | A/A pH > 13  | 0.72 V/cm, 300 mA, 20 min, 4°C | QTM      | Donà et al., 2013         |
|          | S. tuberosum        | Roots, leaves        | 30 G y-ray    | Tris           | A/A pH > 13  | 0.74 V/cm, 300 mA, 15 min, 4°C | %TD/LDR | Nishiguchi et al., 2012   |
| X-ray    | A. thaliana         | Leaves               | 15 G X-ray    | PBS-EDTA       | A/A pH > 13  | 300 mA, 15 min, 4°C | %TD      | Gichner et al., 2008a     |
|          | N. tabacum          | Leaves, apex, Cotyledons | 50 G X-ray | MBS-EDTA | A/N pH > 13 | 1 V/cm, 10 mA, 5 min | %TD      | Koppen et al., 1999       |
|          | O. sativa           | Calli                | 100 G X-ray   | Sörensen(mod)  | NaN pH 8     | 1 V/cm, 8 min | %TD      | Endo et al., 2012         |
|          | V. faba             | Roots                | 50 G X-ray    | MES saline     | A/N pH > 13 | 1 V/cm, 10 mA, 10 min | %TD      | Koppen and Angelis, 1998  |

(Continued)
| Stress | Species | Tissue | Maximum dose | Nuclei | Comet type | Electrophoresis | Analysis | References |
|--------|---------|--------|--------------|--------|------------|----------------|----------|------------|
|        | V. faba | Roots, leaves | 50 mg/L Ti(OH₂COC) | Tris, C/A | A/A pH > 13 | 1 V/cm, 300 mA, 15 min, 4°C | %TD | (O)TM Radić et al., 2009 |
|        | A. cepa | Roots | 40 μM CdCl₂ | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | TL(0)TM Seth et al., 2008 |
|        | B. monnieri | Roots, leaves | 500 μM CdCl₂ | Tris, C/A | A/A pH > 13 | 0.72 V/cm, 300 mA, 30 min, 4°C | %TD | (O)TM Vajpayee et al., 2006 |
|        | L. sativa | Roots, leaves | 50 μM Cd(NO₃)₂ | Tris | A/A pH > 13 | 0.74 V/cm, 300 mA, 30 min, 4°C | %TD(,TL,O)TM | Monteiro et al., 2012 |
|        | L. luteus | Roots | 1 mM CdCl₂ | Honda | A/A pH > 13 | 1 V/cm, 300 mA, 10 min, 4°C | %TD(,TL,O)TM | Vajpayee et al., 2006 |
|        | N. tabacum | Roots, leaves | 500 μM CdCl₂ | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD | (O)TM Arya and Mukherjee, 2014 |
|        | V. faba | Roots | 200 μM CdCl₂ | Tris | A/A pH > 13 | 300 mA, 15 min | %TD | (O)TM Koppen and Varschaeva, 1996 |
|        | V. unguiculata | Roots | 10 mM CdCl₂ | Tris-MgCl₂ | A/A pH > 13 | 1 V/cm, 300 mA, 20 min, 8°C | TL | (O)TM Arasimowicz-Jelonek et al., 2012 |
|        | C. sativus | Roots | 11 ppm CuSO₄ | Tris-MgCl₂ | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD | (O)TM Yildiz et al., 2009 |
|        | C. esculentum | Roots | 60 ppm CuSO₄ | Tris-MgCl₂ | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD | (O)TM Yıldız et al., 2009 |
|        | M. truncatula | Leaflets | 0.2 mM CuO₂ | Sörensen(mod) | N/N pH > 8.4 | 1 V/cm, 8 min | VS | (O)TM | Faë et al., 2014 |
|        | A. cepa | Roots | 100 μM Pb(NO₃)₂ | Galbraith | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD(,TL,O)TM | Procházková et al., 2013 |
|        | N. tabacum | Roots, leaves | 100 μM Pb(NO₃)₂ | Galbraith | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD(,TL,O)TM | Jiang et al., 2014 |
|        | T. triangulare | Roots | 2.4 mM Pb(NO₃)₂ | Galbraith | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD(,TL,O)TM | Jiang et al., 2014 |
|        | V. faba | Roots | 20 μM Pb(NO₃)₂ | Galbraith | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | %TD(,TL,O)TM | Jiang et al., 2014 |
|        | A. thaliana | Roots | 3 mM B(OH)₃ | PBS-EDTA | N/N pH > 8.4 | 1 V/cm, 25 V, 20 min, 4°C | %TD | (O)TM | Qin et al., 2015 |
|        | Root | 100 μM AlCl₃ | PBS-EDTA | A/A | 0.6 V/cm, 250 mA, 25 min | %TD | (O)TM | Kaur et al., 2014 |

(Continued)
| Stress | Species | Tissue | Maximum dose | Nuclei | Comet type | Electrophoresis | Analysis | References |
|--------|---------|--------|--------------|--------|------------|-----------------|---------|------------|
| A. cepa | Roots   | 200 μM AC\textsubscript{3} | PBS      | A/A pH > 13 | 0.75 V/cm, 300 mA, 25 min, 4°C | TL     | Achary et al., 2008 |
|         |         | 800 μM AC\textsubscript{3} | Tris     | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Achary and Panda, 2010 |
|         |         |            | Tris, C/A | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Achary et al., 2012a |
|         |         |            | Tris      | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Achary et al., 2013 |
| H. vulgare | Leaves | 10 mM Al\textsubscript{3} | Tris     | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Achary et al., 2012b |
| V. faba  | Roots   | 1 mM Cr\textsubscript{3+} | Honda    | A/A pH > 13 | 1 V/cm, 300 mA, 10 min, 4°C | %TD, TL, (QTM) | Koppen and Verschaeve, 1996 |
| Oxanions | A. cepa | Roots   | 200 μM Cr\textsubscript{3+} | Tris     | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Patnaik et al., 2013 |
| P. sativum | Roots, leaves | 2 g/L K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} | PBS-EDTA | A/A pH > 13 | 0.74 V/cm, 15 min | %TD, (QTM) | Rodriguez et al., 2011 |
| V. faba  | Roots, leaves | 100 μM Sn\textsubscript{2}O\textsubscript{4} | PBS-EDTA | A/A pH > 13 | 0.75 V/cm, 300 mA, 15 min, 4°C | (QTM)  | Liman et al., 2008 |
| A. cepa  | Roots, leaves | 30 mg/L Sn\textsubscript{2}O\textsubscript{4} | Tris-NaCl | A/A pH > 13 | 25 V, 300 mA, 45 min | %TD, TL, (QTM) | Boccia et al., 2013 |
| A. cepa  | Roots   | 100 ppm In\textsubscript{2}O\textsubscript{3} | Tris-MgCl\textsubscript{2} | A/A pH > 13 | 2.31 V/cm, 25 V, 20 min, 4°C | VS     | Ciğerci et al., 2015 |
| A. cepa  | Roots   | 100 ppm Bi\textsubscript{2}O\textsubscript{3} | Tris-MgCl\textsubscript{2} | A/A pH > 13 | 1 V/cm, 25 V, 20 min, 4°C | VS     | Liman, 2013 |
| L. esculentum | Roots | 2 mg/ml NO NPs | Galbraith, C/A | A/A pH > 13 | 0.7 V/cm, 300 mA, 30 min, 4°C | VS     | Faisal et al., 2013 |
| N. tabacum | Roots, leaves | 10 mg/L TiO\textsubscript{2} NPs | Tris-MgCl\textsubscript{2} | A/A pH > 13 | 25 V, 300 mA, 20 min, 4°C | %TD, VS | Santos et al., 2013 |
| Quantum dots | M. sativa | Cell suspension | 100 nM CdSe/ZnS QDs | MES CaCl\textsubscript{2}/FPG, EndoIII | A/N pH > 13 | 25 V, 10 mA, 10 min, 4°C | VS | Santos et al., 2013 |
| Dyes | A. cepa | Roots | dyes of Petunia and Gailardia | PBS, Tris | A/A pH > 13 | 0.7–0.75 V/cm, 300 mA, 20–25 min, 4°C | %TD, TL, %HDNA, (QTM) | Watharkar and Jadhav, 2014 |
| Pesticides | A. cepa | Roots | 100 ppm chlorpyrifos | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | VS | Türköğlu, 2012 |
|         |         | 100 ppm fenbuconazole | PBS | A/A pH > 13 | 0.7–0.75 V/cm, 300 mA, 20–25 min, 4°C | %TD, TL, %HDNA, (QTM) | Watharkar and Jadhav, 2014 |
|         |         | 100 ppm fenamiphos | PBS | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | VS | Liman et al., 2011 |
| Stress | Species | Tissue | Maximum dose | Nuclei | Comet type | Electrophoresis | Analysis | References |
|--------|---------|--------|--------------|--------|------------|----------------|----------|------------|
| I. balsamina | Leaves | 80 ppm imazethapyr | Tris-MgCl2 | A/A pH > 13 | 1 V/cm, 20 min, 4°C | VS | Liman et al., 2015 |
| O. sativa | Calli | 145 mM feranimol | Sörensen(mod) | A/A pH > 13 | 0.66 V/cm, 230 mA, 10 min, 4°C | LDR, VS | Poli et al., 2003 |
| P. vulgaris | Roots | 5 mg/L aphidicolin | PBS-EDTA | NN pH 8.4 | 6 min | (Q)TM | Kwon et al., 2013 |
| Polyhalogenated | A. cepa | Roots | 100 mg/L bromoform, 200 mg/L chloroform | Tris-MgCl2 | A/A pH 12.3 | 1 V/cm, 25 V, 20 min, 4°C | VS | Cerck et al., 2010 |
| N. tabacum | Roots, leaves | 4.8 mM CBA, 1 mM DCBA, 0.48 mM TCBA | PBS | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | (O)TM | Gichner et al., 2003b |
| CONTAMINATED MATRICES | A. cepa | Roots | fly ash mixtures (100%) | Tris | A/A pH > 13 | 0.7 V/cm, 300 mA, 20 min, 4°C | %TD,(O)TM | Chakraborty et al., 2009 |
| C. occidentalis | Leaflets | soil containing fly ash | Tris | A/A pH > 13 | 0.7 V/cm, 300 mA, 20 min, 4°C | %TD,(O)TM | Ghosh et al., 2012b |
| V. zizanioides | Roots | fly ash mixtures (100%) | Tris | A/A pH > 13 | 0.7 V/cm, 300 mA, 20 min, 4°C | %TD | Love et al., 2009 |
| Effluents | A. cepa | Roots | 100% acid mine drainage | PBS-EDTA | A/A pH > 13 | 25 V, 300 mA, 20 min, 4°C | VS | Defaveri et al., 2009 |
| L. minor | Plant | effluent waters | Tris | A/A pH > 13 | 1 V/cm, 300 mA, 20 min, 4°C | (Q)TM | Radić et al., 2010 |
| Leachates | A. cepa | Roots | 100% landfill leachate | Tris | A/A pH > 13 | 1 V/cm, 300 mA, 20 min, 4°C | %TD(T)M | Radić et al., 2013 |
| E. fetida | Shoots | 100% landfill leachate | Tris | A/A pH > 13 | 1 V/cm, 300 mA, 20 min, 4°C | %TD(T)M | Radić et al., 2013 |
| T. repens | Shoots | 100% landfill leachate | Tris | A/A pH > 13 | 1 V/cm, 300 mA, 20 min, 4°C | %TD(T)M | Radić et al., 2013 |
| Metals | N. tabacum | Leaves | metal-polluted soil | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 25 min, 4°C | (Q)TM | Gichner et al., 2006 |
| S. tuberosum | Leaves | metal-polluted soil | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 15 min, 4°C | (Q)TM | Gichner et al., 2006 |
| T. repens | Leaves | metal-polluted soil | PBS | A/A pH > 13 | 300 mA, 15 min, 4°C | %TD, TL, (Q)TM | Bhat et al., 2011 |
| Chemicals | N. tabacum | Leaves | PCB-polluted soil | Tris | A/A pH > 13 | 0.72 V/cm, 300 mA, 30 min, 4°C | (Q)TM | Gichner et al., 2007 |
| Radiation | A. cepa | Roots | 190000 Bq/kg 226Ra soil | Tris | A/A pH > 13 | 0.65 V/cm, 230 mA, 20 min, 4°C | (Q)TM | Saghirzadeh et al., 2008 |
| Gases | P. tremuloides | Leaves | 1.5 × O3, 200 ppm above normal CO2 | PBS-EDTA | A/A pH > 13 | 1 V/cm, 300 mA, 30 min, 4°C | %TD | Tai et al., 2010 |
| PHYTOCOMPONDS | A. cepa | Roots | 100 mg/l T. turcica extract | Tris-MgCl2 | A/A pH > 13 | 1 V/cm, 20 min, 4°C | VS | Ciğerci et al., 2014 |
| L. sativa | Roots | 200 μM epinodosin | Tris | A/A pH > 13 | 0.74 V/cm, 300 mA, 25 min, 4°C | %TD(T)M | Ding et al., 2010a |
| Stress           | Species                  | Tissue   | Maximum dose | Nuclei         | Comet type         | Electrophoresis                  | Analysis | References                  |
|------------------|--------------------------|----------|--------------|----------------|--------------------|----------------------------------|----------|-----------------------------|
|                  |                          |          |              |                |                    |                                  |          |                             |
| **OSMOSSTRESSORS** |                          |          |              |                |                    |                                  |          |                             |
|                  | A. thaliana              | Seedlings| 200 mM NaCl  | PBS-EDTA       | N/N pH 8.4         | 1V/cm, 12 mA, 5 min          | %TD     | Roy et al., 2013          |
|                  | M. truncatula            | Roots    | 50 g/L PEG 6000 | Sörensen(mod)   | N/N pH 8.4         | 1V/cm, 8 min                  | VS       | Confalonieri et al., 2014  |
|                  | O. sativa                | Seedlings| 100 mM NaCl  | PBS-EDTA       | A/A pH 13           | 0.72 V/cm, 20 min, 4°C        | VS       | Macovei and Tuteja, 2013   |
| **CONTROL MUTAGENS** |                          |          |              |                |                    |                                  |          |                             |
|                  | A. cepa                  | Roots, leaves| 8 mM EMS    | Tris, C/A      | A/A pH 13          | 0.72 V/cm, 300 mA, 20 min, 4°C | %TD     | Bandypadhay and Mukherjee, 2011 |
|                  | A. thaliana              | Seedlings| 50 mg/L BLM  | PBS-EDTA       | A/A pH 10          | 1V/cm, 12 mA, 5 min          | %TD     | Böhmderfer et al., 2011    |
|                  |                          |          |              |                |                    |                                  |          |                             |
|                  | B. monnieri              | Roots, leaves| 2 μg/mL BLM | Tris           | A/A pH 13          | 0.72 V/cm, 300 mA, 30 min, 4°C | (QTM)   | Vajpayee et al., 2006      |
|                  | C. capillaris            | Leaves   | 2 mM MH      | Tris           | A/A pH 13          | 1.3 V/cm, 340 mA, 15 min, 4°C | TL,(O)TM,VS | Kwasniewska et al., 2012 |
|                  | H. vulgare               | Roots    | 200 mg/L BLM | Sörensen(mod)  | N/N pH 8.4         | 1.0 V/cm, 120 mA, 40 min, 4°C  | %TD,VS  | Georgieva and Stoikov, 2008 |
|                  | L. perenne               | Leaves   | 60 mM EMS    | Tris-EDTA      | A/A pH 13          | 0.72 V/cm, 300 mA, 5 min, 4°C  | %TD     | Pourrut et al., 2015       |
|                  | M. giganteus             | Leaves   | 60 mM EMS    | Tris-EDTA      | A/A pH 13          | 0.72 V/cm, 300 mA, 5 min, 4°C  | %TD     | Pourrut et al., 2015       |
|                  | N. tabacum               | Roots, leaves| 8 mM EMS    | Tris, C/A      | A/A pH 13          | 0.72 V/cm, 300 mA, 20 min, 4°C | %TD     | Bandypadhay and Mukherjee, 2011 |
|                  | Roots                    |          | 10 mM EMS, 20 μM H₂O₂ | Tris, C/A | A/A pH > 13       | 0.72 V/cm, 300 mA, 30 min, 4°C | (QTM)   | Gichner, 2003b            |
|                  | Leaves                   |          | 4 mM EMS, 0.4 mM ENU, 0.5 mM MH | Tris | A/A pH > 13       | 0.72 V/cm, 300 mA, 30 min, 4°C | (QTM)   | Gichner, 2003a            |
|                  | P. x hybrida             | Roots, leaves| 3 mM EMS, 0.4 mM ENU | Sörensen(mod) | A/A pH > 13       | 16V, 300 mA, 30 min, 4°C        | %TD,(Q)TM,VS | Juchimiuk et al., 2006 |
|                  | P. patens                | Protonema| 50 mg/L BLM  | PBS-EDTA       | A/N pH > 13        | 1V/cm, 12 mA, 3 min            | %TD     | Holá et al., 2013         |
|                  |                          |          | 50 mg/L BLM  | PBS-EDTA       | N/N pH 8.4         | 1V/cm, 12 mA, 3 min            | %TD     | Holá et al., 2013         |

(Continued)
| Species          | Tissue    | Maximum dose | Electrophoresis | Analysis | Comet type | References               |
|------------------|-----------|--------------|-----------------|----------|------------|--------------------------|
| S. tuberosum     | Roots, leaves | 50mg/L       | PBS-EDTA       | N/N      | %TD        | Kamisugi et al., 2012    |
|                  |           | 1V/cm, 12mA, 5min |                |          |            |                          |
| S. tuberosum     | Leaves    | 8mM EMS      | Tris            | A/A      | %TD        | Gichner et al., 2008a    |
| T. repens        | Leaves    | 60mM EMS     | Tris-EDTA      | A/A      | %TD        | Pourrut et al., 2015     |
| V. faba          | Roots     | 1mM MMS, 1mM EMS | Tris            | N/N      | %TD        | Koppen and Verschaeve, 2001 |
| V. faba          | Leaves    | 60mM EMS     | Tris            | N/N      | %TD        | Pournou et al., 2015     |
| V. unguiculata   | Leaves    | 10mM EMS     | Tris            | N/N      | %TD        | (O)TM Gichner et al., 2003 |
| Various species  | Leaves    | 10mM EMS     | Tris            | N/N      | %TD        | Pourrut et al., 2015     |
| Various species  | Roots, leaves | 5mM EMS    | Tris            | A/A      | %TD        | Kamisugi et al., 2012    |
|                  |           | 1V/cm, 12mA, 5min |                |          |            |                          |
| O. sativa        | Leaves    | 500mg/BrdU, 1M FdU | MBS-EDTA       | N/N      | %TD        | Endo et al., 2012        |
| P. x hybrid      | Leaves    | 1V/cm, 12mA, 5min | Tris            | A/A      | %TD        | Enseit and Collins, 2015 |
| P. patens        | Leaf calli | 1V/cm, 12mA, 5min | Tris            | A/A      | %TD        | Donà et al., 2014        |

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Among these, the alkaline comet assay has been widely used to evaluate DNA damages induced by γ-rays. Using alkaline comet assay, Koppen and Angelis (1998) demonstrated that γ-rays induce a linear increase of DNA content in the comet tail of irradiated *V. faba* plants. Endo et al. (2012) reported that γ-ray exposure in calli of *Oryza sativa* resulted in a dose-dependent increase of DSBs, as shown by neutral comet assay. Recently, Enseit and Collins (2015) studied the effect of low dose radiations on DNA repair mechanisms using alkaline comet assay. They identified two phases of DNA repair after acute exposures of 5
and 15 Gy ("rapid" and "slow" phases). With lower exposures (2 Gy and lower), they also highlighted that "rapid" repair was so fast that it was difficult to detect.

Concerning radioactive contaminations, Saghirzadeh et al. (2008) successfully demonstrated that very high levels of natural radioactivity (e.g., by accumulation of $^{226}$Ra) presented by soils were significantly genotoxic to *A. cepa* roots, with DNA damages measured by comet assay and compared to the effects of increasing $\gamma$-ray doses.

### Metals

Most of the contaminated sites worldwide are contaminated with heavy metals. In Europe, heavy metals contaminated almost 50% of the investigated sites (Panagos et al., 2013). Exposure to metals may induce a variety of direct and indirect phytotoxic effects (e.g., Silva et al., 2010). In general metals induce more severe symptoms in roots than in leaves, since roots are in direct contact with the soil and generally with the toxic contaminant.

The first comet assays evaluating metal genotoxicity in plants were pioneered by Koppen and Verschaeye (1996) which studied chromium (Cr) and cadmium (Cd) genotoxicity in *V. faba*. These authors showed a dose-dependent increase in DNA damage. More recently, Cd-induced DNA degradations were also observed in *Trifolium repens* (Bhat et al., 2011), *Lactuca sativa* (Monteiro et al., 2012), *Lupinus luteus* (Arasimowicz-Jelonek et al., 2012), *Vigna unguiculata* (Amirthalingam et al., 2013), *N. tabacum* (Tkalec et al., 2014), *V. faba* and *A. cepa* (Arya and Mukherjee, 2014). However, dose-dependent responses were not clearly observed in these studies. This could be explained by the fact that these authors lead hydroponic studies and used very high and environmental-unrealistic concentrations of cadmium. Monteiro et al. (2012) suggested that these high concentrations could induce Cd-DNA adducts that lead to DNA-DNA/DNA-protein cross-links, and/or formation of longer DNA fragments, and/or impairment of DNA repair mechanisms, which could explain these results. Interestingly, the only study using soil spiked with environmental-realistic concentrations of cadmium (Hattab et al., 2010), demonstrated a dose-dependent increase in DNA damages in *P. sativum*. Tkalec et al. (2014) and Amirthalingam et al. (2013) also used the comet assay to understand Cd-induced genotoxicity mechanisms. They suggested the implication of oxidative stress while Arasimowicz-Jelonek et al. (2012) showed that scavenging the endogenous nitric oxide (NO) pool during Cd stress, despite reducing the programmed cell death, did not affect the degree of DNA damages evidenced by comet assay. Recently, comet assay was used to investigate the difference of sensitivity to Cd exposure of *A. cepa* and *V. faba* (Arya and Mukherjee, 2014). The results indicated that exposure to Cd induced slight dose-dependent increase in chromosomal aberrations, DNA fragmentation and micronucleus frequency in both *A. cepa* and *V. faba*. However, *V. faba* appeared more sensitive than *A. cepa* toward Cd-induced genotoxicity, which was correlated to the increased level of oxidative stress in root tissues.

Along with Cd, aluminum (Al) genotoxicity has been the most studied during the last years. Achary et al. (2008, 2012a) and Achary and Panda (2010) demonstrated dose-dependent DNA damage induced by Al exposure on *A. cepa* roots. These results were confirmed later on *Hordeum vulgare* (Achary et al., 2012b) and *Andropogon virginicus* (Ezaki et al., 2013). These studies also highlighted the implication of oxidative stress in Al genotoxicity.

Comet assay was also used to investigate the mechanisms of Al genotoxicity, underscoring the role of cell wall-bound NADPH in the Al oxidative burst-mediated (Achary et al., 2012a), and the role of signal transduction mediated by Ca$^{2+}$ (Achary et al., 2013) and MAP Kinases (Panda and Achary, 2014) in Al-induced cell death and DNA damage. Interestingly, these authors also described the occurrence of adaptation responses that involved oxidative stress, and that root cells conditioned with low doses of Al (<10 $\mu$M Al$^{3+}$) developed adaptive responses and protection mechanisms against genotoxic effects of the mutagenic agents methylmercuric chloride (MMCl) and ethyl methanesulfonate (EMS) (Achary et al., 2013). Moreover, the role of DNA damage in Al-dependent root growth inhibition was also investigated in *A. thaliana* mutants (Rounds and Larsen, 2008; Nezames et al., 2012).

The phytotoxicity of lead (Pb) including genotoxic aspects was reviewed by Pourrut et al. (2011a). Using comet assay, Gichner et al. (2008c) were the first to demonstrate dose-dependent Pb-induced DNA damage in *N. tabacum* in hydroponic and soil experiments. These results were confirmed on *Talinum triangulare* roots and correlated with Pb-induced oxidative stress (Kumar et al., 2013). However, both studies used very high and environmentally-unrealistic concentrations of Pb. More interestingly, dose-dependent Pb-induced DNA damage were also observed with lower and environmentally-realistic concentrations of Pb (<20 $\mu$M Pb) in *V. faba* plants (Pourrut et al., 2011b). Moreover, these authors also confirmed the role of oxidative stress in this damage process, since co-incubation with antioxidant vitamin E or the NADPH-oxidase inhibitor dephenylene iodonium inhibited DNA damage and micronuclei formation in exposed roots (Pourrut et al., 2011b). Recently, two studies performed on *A. cepa* confirmed the role of oxidative stress in lead-induced genotoxicity and that DNA damages are also tightly linked to the cell cycle (Jiang et al., 2014; Kaur et al., 2014).

Similarly, the micronutrient copper (Cu) was shown to induce significant DNA damages in *A. cepa* roots (Yildiz et al., 2009; Qin et al., 2015). Very high concentrations of copper chloride also increased DNA fragmentations in *P. sativum* roots but not in leaves (Hattab et al., 2010). Similarly to the above-cited metals, Cu-induced DNA damages were associated with cytotoxic damages involving oxidative stress in *Lycopersicon esculentum* and *Cucumis sativus* roots (Ileri et al., 2011) and other chromosome aberrations in *A. cepa* roots (Yildiz et al., 2009). Recently, Faè et al. (2014) used the neutral comet assay to demonstrate the overexpression efficiency of the DNA repair gene MtTdp2a for enhancing plant tolerance to Cu exposure in *Medicago truncatula* mutants.

By using the comet assay, Lin et al. (2008) proved that arsenate (10 $\mu$M) induced DNA damages in *V. faba* leaves.
and roots, in a dose-dependent manner and that these effects were associated with oxidative stress. Sturchio et al. (2011) confirmed As genotoxicity in V. faba roots grown on sandy and clay-loamy soil spiked with arsenate. In the same species, Boccia et al. (2013) combined the comet assay with infrared (FTIR), and near infrared (FTNIR) spectroscopy, to show that arsenate (20 and 30 mg/L) induced DNA damages which were associated with structural changes of different functional groups, suggesting the possible replacement of phosphate by arsenate in DNA.

The plant comet assay also contributed to clarify the effects of several other metals in plant DNA damages (Table 1). For example, Radev et al. (2009) demonstrated that the rare metal thallium (Tl), released to the environment as a by-product of Fe and Zn refining processes, induces DNA damages together with oxidative damages in V. faba seedlings. The comet assay was also helpful in demonstrating that boron (B) toxicity mechanism in plants involves DSBs and possibly replication blocks, with plant condensin II playing a critical role in DNA damages repair (Sakamoto et al., 2011). Rodriguez et al. (2011) and Rodriguez (2011) used a battery of genotoxic and cytotoxic biomarkers to assess Cr (VI) toxicity in pea, and were able to correlate Cr (VI)-induced DNA damages (demonstrated by comet assay) with cell cycle arrest at the G2/M checkpoint and with clastogenicity assessed by flow cytometry (Rodriguez, 2011, PhD thesis). Moreover, Patnaik et al. (2013) showed by alkaline comet assay that induction of DNA damage by Cr (VI) was dose-dependent in A. cepa. However, in plants exposed to 1-day treatment followed by 4-day recovery, no effects were found by comet assay. On the same plant species, cobalt (Co) was shown to induce significant DNA damages (Yuldız et al., 2009).

Besides some more established physiological analyses, the comet assay has also been conducted to determine the differential toxic effects affecting different plant organs. Procházková et al. (2013) showed that in N. tabacum zinc (Zn) induces higher DNA damages in roots compared to leaves. This differential effect was possibly attributable to the higher accumulation of Zn (II) in roots, compared to shoots. Tkalec et al. (2014) also observed these effects in N. tabacum. However, these authors also showed that, when Zn was added in the culture medium in combination with Cd, this metal conversely exhibited a protective effects against Cd-induced DNA damages.

It is worth noting that the interest of using the comet assay as a reliable biomarker on ecotoxicological assays is increasing, and Bandyopadhyay and Mukherjee (2011) applied both acellular and cellular comet tests to compare A. cepa and N. tabacum as toxicity models in rapid monitoring Cd-induced genotoxicity. Monteiro et al. (2012) used a battery of tests including the comet assay, to determine differences associated with organ dependence in Cd toxicity. The authors used Lactuca sativa and integrated cytostaticity/genotoxicity and oxidative stress data, where parameters measured by the comet assay (e.g., tail moment) were demonstrated to be relevant genotoxicity biomarkers. Despite still restricted to a few number, some studies have already used plant comet in field ecotoxicology assays of soils contaminated with metals (see Section “Contaminated Matrices” below).

Nanocompounds

Plant comet assays are also increasingly used to assess the phytotoxicity of small-scale materials (Table 1), e.g., nanomaterials and in particular nanoparticles (NPs). Nanomaterials possess unique properties suitable for a wide range of industrial applications. For this reason and due to their intense uses and subsequent release to the environment, they are currently classified as emerging contaminants. One example of emerging nanomaterials are carbon nanotubes, that depending on the physical properties can pose cytotoxicity to mammalian and plant cells (Ghosh et al., 2011). Ghosh et al. (2011, 2015a) demonstrated a correlation between DNA strand breaks and the concentration of multi-walled carbon nanotubes in A. cepa, supporting the genotoxic potential of this type of nanomaterials.

The increasing amount of NPs in groundwater and soil has raised environmental concerns regarding their putative toxicity and fate through food chains. A large group of NP contaminants include toxic or reactive metals NPs. One of the most relevant pioneer studies of NPs genotoxicity in plants was done with TiO2 NPs in A. cepa (Ghosh et al., 2010). In this study the comet assay was used to assess DNA damages and this endpoint was combined with oxidative stress endpoints (e.g., malondialdehyde level). Moreover, in A. cepa roots, TiO2 NPs induced DNA damages confirmed by comet assay and correlated with the occurrence of chromosomal aberrations (Pakrashi et al., 2014).

Silver nanoparticles (AgNPs) were shown to induce DNA damages in A. cepa and N. tabacum with more pronounced effects in roots than in shoots (Ghosh et al., 2012a).

Recently, using higher NPs concentrations, Thiruvengadam et al. (2014) also demonstrated a dose-dependent increase in DNA damages in Brassica rapa ssp. rapa, and this result was confirmed by DNA laddering and TUNEL assays.

Bismuth (III) oxide NPs increased the nuclear DNA damages in A. cepa plants. These data supported the concomitant observation of chromosomal aberrations and mitotic aberrations in the same tissues (Limam, 2013).

The alkaline comet assay showed an increase of DNA damages in tomato seedlings exposed to NiO-NPs up to 2 mg/ml (Faisal et al., 2013). In this study the authors also used the plant comet assay test to assess the percentage of necrotic and apoptotic cells, however, these conclusions must be regarded carefully as the validity of the comet assay in identifying apoptotic cells remains a matter of discussion (Collins et al., 2008).

Indium (III) oxide and tin (IV) oxide is a mixture widely used in industrial coating. A significant increase in DNA damages was recently observed of A. cepa root meristematic cells exposed to doses up to 100 ppm of indium tin oxide suspension (Gığerci et al., 2015).

Besides metal oxide NPs, quantum dots form another type of nanomaterials increasingly prevalent in the environment. Quantum dots are nanomaterials used in electronics which possess semiconducting properties, composed for example of arsenic (As), selenium (Se) and tellurium (Te) in various proportions. Despite their increasing prevalence in the environment, the toxicity of quantum dots in plants is largely unknown. In a pioneer study, Santos et al. (2013)
used a battery of tests and gene expression related with DNA repair, and demonstrated that 10 mM 3-mercaptopropanoic coated-CdSe/ZnS quantum dots were cytotoxic and genotoxic to *Medicago sativa* cells. In this and other pioneer studies, the comet assay can play a pivotal role as a tool to assess environmental impacts of suspected emerging nanocontaminants.

**Organic Pollutants**

Several researchers have used the comet assay to monitor DNA damages induced in plants by numerous organic pollutants (Table 1). The most common organic chemical contaminants include reactive compounds, e.g., alkylating agents, azo dyes, cyclic aromatic hydrocarbons and chemicals incorporated in pesticides and herbicides.

The comet assay was recently used to better understand the role of homologous recombination and genome stability during DNA replication. Comet assay was used to study, in alfalfa, broad bean, lentil, miscanthus, onion, potato, tobacco, sugar beet and wheat, how different agents including ethyl methanesulfonate (EMS) and/or H2O2 induce DNA damages (Gichner et al., 2008a; Bandyopadhyay and Mukherjee, 2011; Pourrut et al., 2015). Due to their dose-dependent genotoxic effects, EMS and H2O2 became largely used as positive controls in plant comet assays, providing further robustness to the assay (Gichner et al., 2008a; Bandyopadhyay and Mukherjee, 2011; Pourrut et al., 2015). Similarly, the dose-dependent induction of DNA damages by compounds such as N-methyl-N-nitroso-urea (MNU), methyl methanesulfonate (MMS) and mitomycin C (MMC) (e.g., Menke et al., 2001; Juchimiuk et al., 2006) supported the wide use of these compounds as positive controls.

Azo dyes are important xenobiotic compounds, largely used in textile industry. Their putative genotoxicity was recently demonstrated in *Petunia grandiflora* and *Gaillardia grandiflora* by comet assay, in a pioneer study of plant–plant association for phytoremediation involving the treatment of textile dyes (Watharkar and Jadhav, 2014). Recently, it was demonstrated that bromoform (which may occur during disinfection processes of water) and chloroform (>25 μg/mL) increased chromosome aberrations and DNA damages, this last one assessed by comet assay (Khallef et al., 2013). Bromoform may occur during disinfection processes of water. Chloroform has been shown to induce DNA oxidative damages and single and double strand breaks in the wild moss *Physcomitrella* lines and in the *lig4* mutant (Holá et al., 2013). Similarly, MMC induced a dose-dependent increase in DNA damages in *Arabidopsis* plants (Menke et al., 2001).

**Contaminated Matrices**

Despite the promising data concerning the robustness and suitability of the comet assay for screening metal-induced DNA damages in plant cells, its use to assess the genotoxicity of poly-contaminated matrices, including samples of contaminated soils, of leakages or fly ashes, remains scarce (Table 1). In a pioneer study, Gichner et al. (2006) used the alkaline comet assay to demonstrate DNA damages in both *Phaseolus vulgaris* roots used treated by two herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and Dicamba (3,6-dichloro-2-methoxybenzoic acid). These results were confirmed in the same study by RAPD analysis. Recently, Liman et al. (2015) also observed a dose-dependent DNA degradation induced by the imidazolinone herbicide Imazethapyr in *A. cepa* roots.

Antibiotics were also shown to induce DNA damages in plant cells. For example, the cytostatic effects of the antibiotic bleomycin (a DNA damaging glycopeptide) were demonstrated in plants, e.g., barley (Georgieva and Stoilov, 2008; Stoilov et al., 2013). Bleomycin also induced DNA oxidative damages and single and double strand breaks in the wild moss *Physcomitrella* lines and in the *lig4* mutant (Holá et al., 2013). Similarly, MMC induced a dose-dependent increase in DNA damages in *Arabidopsis* plants (Menke et al., 2001).
et al., 2010). Importantly, the same group (Radič et al., 2013) found comparable responses in fish and Lemma minor regarding DNA damage and oxidative stress, after exposure to polluted surface water contaminated by a fertilizer factory effluent rich in fluorides, metals, and polycyclic aromatic hydrocarbons. The authors highlighted that their results imply that conventional chemical analysis should be extended to genotoxicity/toxicity biological assays to better predict potential health hazard.

Fly ashes are generated during combustion, and include fine particles, with different sizes, rising to the atmosphere. Their complex constitution raised questions on their genotoxicity to animals and plants. Love et al. (2009) demonstrated, based upon comet assay results, that higher levels of DNA damages were found in leaves of Cassia occidentalis exposed to fly ash, compared to non-exposed controls. The authors suggested that these DNA damages might be associated with foliar concentrations of As and Ni absorbed from the fly ash. Ghosh et al. (2012b) studied the genotoxicity in A. cepa of soil samples contaminated with metal-rich fly ashes from a thermal power plant in India and concluded that the observed DNA damages could be correlated to the presence of toxic metals. Also, Chakraborty et al. (2009) studied the genotoxic effects of fly ash comparing the comet assay and the Allium test in this model species. The authors supported the combination of these two techniques in monitoring assays. The same group used the comet to validate the relevance of Vetiveria zizanioides as a good candidate for remediation of fly ash dumpsites (Chakraborty and Mukherjee, 2011). They demonstrated this plant could grow in the presence of fly ash without any genotoxic effects in comparison to A. cepa which exhibited a very high DNA degradation (>80%). Later, this research group used comet assay on A. cepa to monitor the remediation efficiency of V. zizanioides on fly ash amended soils (Ghosh et al., 2015b). They showed that this plant was able to strongly mitigate the genotoxic potential of these soils. These results were also confirmed by a reduction in micronuclei formation, binucleate cells and chromosomal aberrations.

The effects of air contaminants on plant DNA-damages have also been studied in the last years. For example, Populus tremuloides clones exposed to air enriched with O₃ alone, or CO₂ + O₃ showed increased DNA damages levels above background as measured by the comet assay, but these effects were genotype dependent (Tai et al., 2010).

Phytocompounds

A wide number of phytocompounds (including alkaloids, phenolic compounds, glycosides, flavonoids, anthocyanins, etc) may have cytotoxic and genotoxic effects or have protective roles against stress conditions in a wide number of species, including humans. The way phytocompounds influence oxidative stress balances, and regulate programmed cell death pathways and cell cycle checkpoints, support their wide therapeutic use (e.g., Ascenso et al., 2013; Ferreira de Oliveira et al., 2014). Recently, the interest of using comet assay to monitor genotoxic effects of some phytocompounds on other plant species has emerged (Table 1). For example, Petriccione and Ciniglia (2012) demonstrated the occurrence of a dose-dependent accumulation of DNA damages in Raphanus sativus (radish) radicles treated with Juglans regia husk water extracts. It should be noted that the authors stressed the need of performing accurate and appropriate statistical evaluations of comet results, an emerging topic of discussion. Ciğerci et al. (2014) also used alkaline comet assay to demonstrate the genotoxicity of Thermopsis turcica extracts on A. ceapa roots. They showed dose-dependent DNA damages which were confirmed by RAPD profile analysis.

The alkaloid narciclasine (extracted from N. tazetta) was recently shown to inhibit plant growth of Orzya sativa, A. thaliana, Brassica rapa or Lactuca sativa (Hu et al., 2014). The comet assay, complemented with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, showed a narciclasine dose-effect response in lettuce seedlings, and this triggered DNA damages may involve increased oxidative stress (Hu et al., 2014). Contrarily, anthocyanins protected DNA integrity (detected by comet assay) in Arabidopsis plants during prolonged exposure to high-light (1300 mmol/m²/s) (Zeng et al., 2010).

Epinodosin, and rabdosin B, diterpenoids isolated from Isodon japonica, exhibited a biphasic dose-dependent effect on Lactuca sativa root growth. The inhibitory effects of both compounds found at higher doses was paralleled with an increase of DNA damages and an inhibition of root cell mitotic activity or retardation of the cell cycle, respectively (Ding et al., 2010a,b). Other terpenes (saponins) extracted from Medicago sativa were shown to induce SSBs and DSBs in Populus alba cell cultures (Paparella et al., 2015). Very interestingly, these authors demonstrated that for all 11 tested saponins, neutral comet assay resulted in similar DSBs patterns, indicating a general response to saponin-induced genotoxic stress, not related to the specific structure of these molecules. Differently, the evaluation of DNA damages performed with alkaline comet assay provided distinct profiles depending on the tested saponin.

Comet assay was also used to evaluate the effect of the phytohormone salicylic acid. Interestingly, Yan et al. (2013) demonstrated that salicylic acid can generate DNA damages in the absence of a genotoxic agent in A. thaliana, supporting that activation of DNA damage responses is an intrinsic component of the plant defense responses.

Comet Assay and Putative Genetic Associations

The comet assay has contributed to elucidate the DNA repair mechanisms involved in the response to external stress factors. A variety of methodologies can be used to investigate DNA repair mechanisms in plants (Azqueta et al., 2009), the most common being the study of plants exposed to DNA mutagens and comparison of plant strains deficient in specific DNA repair pathways. Ionizing radiation and a variety of genotoxins specifically induce DSBs and are frequently analyzed together with the action of radiomimetic compounds, such as bleomycin (e.g., Menke et al., 2001; Waterworth et al., 2009; Böhmdorfer et al., 2011; Wang et al., 2014), zeocin (Nishiguchi et al., 2012), or MMS (e.g., Menke et al., 2001; Vajpayee et al., 2006; Waterworth et al., 2009). Other mutagens frequently used to study DNA repair and strand breaks include agents that induce point mutations,
dsb

HEB2

that DSBs are a cause of B toxicity and that condensin II reduces maintenance of chromosome structure. These results suggested B induced DNA damages and affected the expression of genes involved in Homologous Recombination (HR) might occur during S phase and stimulate HR in fas mutants. Also, levels of formed DSBs were compared in rice wild type plants vs. an mutant plants quickly repaired the DNA damage produced by bleomycin and γ-rays, and that they showed preferential use of non-conservative mechanisms. Moreover, in Arabidopsis knock-down strains of DNA ligase I, Waterworth et al. (2009) found by neutral comet assay that the LIG1 knock-down strains were less efficient in the repair of DSBs compared to wild-type, suggesting that the AtLIG1 gene is involved also in DSB repair pathway.

Several transcripts related with DNA damage pathways, DNA replication, and repair, oxidative stress and cell cycle progression have been identified in plant cells associated with alterations in comet assay profiles. Some of the most relevant studies in wildtype plants are summarized in Table 2. For example, Endo et al. (2006) demonstrated that Arabidopsis fas mutants showed increased levels of DNA DSBs. The authors proposed that the induction of DNA DSBs and enhanced transcription of genes involved in Homologous Recombination (HR) might occur during S phase and stimulate HR in fas mutants. Also, levels of formed DSBs were compared in rice wild type plants vs. an aphidicolin-sensitive phenotype. Without aphidicolin treatment, both WT and osrecql4-2 mutants produced very low levels of DSBs, but these increased in the mutants after treatment (Kwon et al., 2013).

Böhmderfer et al. (2011) studied the involvement of γ-irradiation and MMC induced one protein (GM11), a structural-maintenance-of-chromosomes-hinge domain-containing protein in mechanisms of somatic homologous recombination in Arabidopsis mutant lines. Comet assay demonstrated that the gmi1 mutants had a reduced rate of DNA DSB repair during the early recovery phase after exposure to bleomycin. Also Yao et al. (2013) used the comet assay to show an increase of DNA damage levels in Arabidopsis sdg2 mutants, containing a mutation at SET DOMAII GROUP 2, necessary for global genome wide deposition of histone H3 lysine 4 trimethylation in chromatin. With these results, authors contributed to elucidate the regulation of SDG2-mediated H3K4me3 on chromatin structure and genome integrity in plants.

Sakamoto et al. (2011) studied Arabidopsis mutants (heb1-1 and heb2-1) hypersensitive to excess of boron (B). Excess of B induced DNA damages and affected the expression of HEB1 and HEB2, which encode respectively the CAP-G2 and CAP-H2 subunits of the condensin II protein complex, important in maintenance of chromosome structure. These results suggested that DSBs are a cause of B toxicity and that condensin II reduces the incidence of DSBs (Sakamoto et al., 2011).

Santos et al. (2013) demonstrated in Medicago sativa that exposure to increasing concentrations of MPA-CdSe/ZnS quantum dots, led to an increase of DNA damages, and up-regulated the transcription of the DNA repair enzymes formamidopyrimidine DNA glycosylase, tyrosyl-DNA phosphodiesterase I and DNA topoisomerase I.

Roy et al. (2011, 2013) reported that Arabidopsis atpolΔ mutant lines exposed to UV-B radiation or to high salinity and MMC treatment s showed higher accumulation of DSBs than wild-type plants and a delayed repair of DSBs. This fact suggested the requirement of Pol λ in DSB repair in plants. Gamma irradiated Populus nigra suspension-cultured cells showed increased levels of DNA damage and increase of the transcripts PnRAD51, PnLIG4, PnKU70, PnXRC4, and PnPCNA while PnOGG1 mRNA was repressed (Nishiguchi et al., 2012). On the other hand, Donà et al. (2013) tested genotoxic effects of γ-irradiation and found significant fluctuations on the levels of DSB and different capacities of DNA repair, together with dose-rate-dependent changes in the expression of the genes PhMT2 (encoding for a type 2 metallothionein) and PhAPX (encoding for a cytosolic isofrom of ascorbate peroxidase).

Probing FISH techniques have been successfully applied to comet assay preparations to detect specific DNA lesions, nuclear organizer regions (NORs) and telomeric regions in V. faba (Menke et al., 2000) or 5S/25S rDNA in Crepis capillaris (Kwasniewska et al., 2012).

Salt, drought and osmotic stress are ever more emerging as abiotic defies intimately related with soil overuse and climate changes (e.g., Santos et al., 2002; Brito et al., 2003). Salt stress induction of DNA damages has been explored in e.g., Arabidopsis mutants by Roy et al. (2013) who supported the role of Polλ in DNA damages repair. Salt stress and/or radiation induction of DNA damages was studied in rice by Macovei and collaborators who also evaluated the expression of OsXPB2, OsXPD, OsTFIIS, and OsTFIIS-like genes (Macovei and Tutega, 2013; Macovei et al., 2014). Recently, Balestrazzi et al. (2014) demonstrated in Medicago truncatula plants that a prolonged exposure to osmotic stress can cause unwanted DNA damages, while negatively affected the expression profiles of genes involved in DNA repair, namely MtTdp1 (tyrosyl-DNA phosphodiesterase), top1 (DNA topoisomerase I), MtTFIIS (transcription elongation factor II-S) and MtTFIIS-like. So, despite comet assay has not been consistently applied to these environmental stresses in plants, the available data of their interference with DNA integrity, opens a perspective of their use in the near future. Also, Confalonieri et al. (2014) demonstrated that in Medicago truncatula the MtTdp2α-gene overexpression prevented the accumulation of DSBs in absence or presence of osmotic stress, and that the MtMRE11, MtRAD50 and MtNBS1 genes that are involved in DSB sensing/repair, being up-regulated in the MtTdp2α-overexpressing plants grown under physiological conditions, were no further up-regulated under osmotic stress (Confalonieri et al., 2014).

Conclusions

In this review we have highlighted most relevant studies that used comet assay in plants to study the impact of stress conditions on
plant DNA damages. This work was mostly focused on the most recent major advances in the last five, regarding conventional and emerging contaminants and complex matrices. The recent advances in the use of the plant comet assay to both a larger number of plant species, and a larger number of conditions, support the use of this technique as a robust and sensitive technique to assess DNA damages induced by stress conditions. Data also support that this simple and robust technique may be a powerful tool to complement conventional and -omics tools in situ environmental pollution monitoring. Moreover, new fields of research using plant comet assay are open, not only in environmental studies, but also in plant physiology, as this technique may help elucidating pathways involved in plant development, cell cycle/programmed cell death, or even plant disease resistance. Also, it remains an important field of research deciphering genetic mechanisms underlying processes related with DNA damage/repair, in which comet assay will have undoubtedly a crucial role.

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**TABLE 2 | Genes differentially expressed in comet assay positive plants.**

| Gene   | Gene function                          | Expr. | Stress            | Species          | References                      |
|--------|----------------------------------------|-------|-------------------|------------------|---------------------------------|
| BRCA1  | HR—DSB repair, ATM pathway (DSB-inducible) | Up    | γ-ray             | A. thaliana      | Böhmdorfer et al., 2011         |
|        |                                        |       | BLM              | A. thaliana      | Wang et al., 2014               |
|        |                                        |       | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| CAP-G2 (HEB1) | Tolerance to DSB induction | Up    | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| CAP-H2 (HEB2) | Tolerance to DSB induction | Up    | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| FPG    | BER; removal of oxidized purines        | Up    | CdSe/ZnS quantum dots | M. sativa      | Santos et al., 2013             |
| GM1    | HR—DSB repair, ATM pathway (DSB-inducible) | Up    | γ-ray, BLM, MMC  | A. thaliana      | Böhmdorfer et al., 2011         |
| GR1    | HR—DSB repair, ATM pathway (DSB-inducible) | Up    | BLM              | A. thaliana      | Wang et al., 2014               |
|        |                                        |       | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| KU70   | NHEJ—DSB repair                        | Up    | γ-ray, zeocin    | P. nigra         | Nishiguchi et al., 2012         |
| KU80   | NHEJ—DSB repair                        | Up    | salt stress (NaCl) | A. thaliana    | Roy et al., 2013                |
| LIG4   | NHEJ—DSB repair                        | Up    | γ-ray, zeocin    | P. nigra         | Nishiguchi et al., 2012         |
|        |                                        |       | salt stress (NaCl) | A. thaliana    | Roy et al., 2013                |
| OGG1   | BER; removal of 7,8-dihydro-8-oxoguanine | Down  | γ-ray            | P. nigra         | Nishiguchi et al., 2012         |
| PARP1  | DSB repair (ATM pathway); SSB repair (ATR pathway) | Up    | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| PCNA   | DNA replication and repair              | Up    | γ-ray            | P. nigra         | Nishiguchi et al., 2012         |
| Polβ   | NHEJ; NER in response to UV; DNA replication | Up    | UV-B             | A. thaliana      | Roy et al., 2011                |
| RAD51  | HR—DSB repair, ATM pathway (DSB-inducible) | Up    | γ-ray, zeocin    | A. thaliana      | Böhmdorfer et al., 2011         |
|        |                                        |       | boric acid       | P. nigra         | Sakamoto et al., 2011           |
| RAD51A2 | HR—DSB repair                          | Up    | X-ray            | O. sativa L.     | Endo et al., 2012               |
| TDP1β  | Repair of topoisomerase l-mediated damages | Up    | CdSe/ZnS quantum dots | M. sativa      | Santos et al., 2013             |
| TOP1β  | Remove DNA supercoils; transcription, DNA replication, recombination | Up    | CdSe/ZnS quantum dots | M. sativa      | Santos et al., 2013             |
| XRCC4  | NHEJ—DSB repair                        | Up    | γ-ray, salt stress (NaCl) | P. nigra      | Nishiguchi et al., 2012         |
|        |                                        |       | salt stress (NaCl) | A. thaliana    | Roy et al., 2013                |
| APX    | Detoxification of peroxide              | Up    | CdSe/ZnS quantum dots | M. sativa      | Santos et al., 2013             |
| SOD    | Detoxification of superoxide            | Up    | γ-ray            | M. sativa        | Santos et al., 2013             |
| MT2    | Metal binding, ROS radical neutralization | Up    | γ-ray            | Petunia x hybrid | Donà et al., 2013               |
| CDKA1  | Cell cycle regulation                   | Up    | boric acid       | A. thaliana      | Sakamoto et al., 2011           |
| CYCA2;1 | Cell cycle progression                  | Up    | boric acid       | A. thaliana      | Sakamoto et al., 2011           |

ATM, Ataxia telangiectasia mutated; ATR, ATM and Rad3 related; BER, base excision repair; HR, homologous recombination; NER, nucleotide excision repair; NHEJ, non-homologous end joining; DSB, double strand breaks; A. thaliana, Arabidopsis thaliana; M. sativa, Medicago sativa; O. sativa, Oryza sativa; P. nigra, Populus nigra.
References

Abas, Y., Touil, N., Kirsch-Volders, M., Angenon, G., Jacobs, M., and Fameliea, I. D. (2007). Evaluation of UV damage at DNA level in Nicotiana plumaginifolia protoplasts using single cell gel electrophoresis. Plant Cell Tiss. Organ. Cult. 91, 145–154. doi: 10.1007/s11240-007-9257-9

Achary, V. M., Jena, S., Panda, K. K., and Panda, B. (2008). Aluminium induced oxidative stress and DNA damages in root cells of Allium cepa L. Ecotoxicol. Environ. Saf. 70, 300–310. doi: 10.1016/j.ecoenv.2007.10.022

Achary, V. M., and Panda, B. B. (2010). Aluminium-induced DNA damages and adaptive response to genotoxic stress in plant cells are mediated through reactive oxygen intermediates Mutagenesis 25, 201–209. doi: 10.1039/mutage/gep063

Achary, V. M., Parinandi, N. L., and Panda, B. B. (2012a). Aluminium induces oxidative burst, cell wall NADH peroxidase activity, and DNA damages in root cells of Allium cepa L. Environ. Mol. Mutagen. 53, 550–560. doi: 10.1002/em.21719

Achary, V. M., Parinandi, N. L., and Panda, B. B. (2012b). Oxidative biomarkers in leaf tissue of barley seedlings in response to aluminum stress. Ecotoxicol. Environ. Saf. 75, 16–26. doi: 10.1016/j.ecoenv.2011.08.015

Achary, V. M., Parinandi, N. L., and Panda, B. B. (2013). Calcium channel blockers protect against aluminium-induced DNA damages and block adaptive response to genotoxic stress in plant cells. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 751, 130–138. doi: 10.1016/j.mrgentox.2012.12.008

Amirthalingam, T., Velusamy, G., and Pandian, R. (2013). Cadmium-induced changes in mitotic index and genotoxicity on Vigna anguiculata (Linn.) Walp. J. Environ. Chem. Ecotoxicol. 5, 57–62. doi: 10.5897/JEECE11.008

Angelis, K. J., Dusinska, M., and Collins, A. R. (1999). Single cell gel electrophoresis: detection of DNA damage at different levels of sensitivity. Electrophoresis. 20, 2133–2138

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Deckert, J., Ruciñska-Przybyłowska, J., Deckert, J., and Ruciñska, J. (2012). Nitric oxide implication in cadmium-induced programmed cell death in roots and communities, 401–405.

Chakraborty, R., and Mukherjee, A. (2011). Technical note: vetiver can grow on coal fly ash without DNA damages. Int. J. Phytoremediation 13, 206–214. doi: 10.1080/15226510903535171

Chakraborty, R., Mukherjee, A. K., and Mukherjee, A. (2009). Evaluation of genotoxicity of coal fly ash in Allium cepa root cells by combining comet assay with the Allium test. Environ. Monit. Assess. 153, 351–357. doi: 10.1007/s10661-008-0361-z

Ciąglici, I., Cienki, S., Kargioglu, M., and Konuk, M. (2014). Genotoxicity of Thymus serpyllum turica on Allium cepa L. roots revealed by alkaline comet and random amplified polymorphic DNA assays. Cytotechnology. 10.1007/s10616-014-9835-8. [Epub ahead of print].

Ciąglici, I., Liman, R., Ozgul, E., and Konuk, M. (2015). Genotoxicity of tinn oxide by Allium and Comet tests. Cytotoxicology 67, 157–163. doi: 10.1007/s10616-015-9673-0

Collins, A., Azqueta, A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., et al. (2008). The comet assay: topical issues. Mutagenesis 23, 143–151. doi: 10.1039/mutage/gem051

Confolani, M., Fai, M., Balestrazzi, A., Donà, M., Macovei, A., Valassi, A., et al. (2014). Enhanced osmotic stress tolerance in Medicago truncatula plants overexpressing the DNA repair gene MGT1dpa2 (tynsyl-DNA phosphodiesterase 2). Plant Cell Tiss. Organ. Cult. 116, 187–203. doi: 10.1007/s11240-013-0395-y

Defari, T. M., da Silveira, F. Z., Bortolotto, T., Geremias, R., Zocche, J. I., and Pich, C. T. (2009). “Evaluation of acid mine drainage treatment using Artemia sp. and Allium cepa as bioindicators of toxicity and genotoxicity,” in Joint Conference of the 26th Annual Meetings of the American Society of Mining and Reclamation and 11th Billings Land Reclamation Symposium (Billings). doi: 10.13140/2.1.4068.9929

Ding, L., Jing, H., Qin, B., Qi, L., Li, J., Wang, T., et al. (2010b). Regulation of cell division and growth in roots of Lactuca sativa L. seedlings by the ent-kaurane diterpenoid rhabdosin B. J. Chem. Ecol. 36, 553–563. doi: 10.1007/s10880-010-9783-5

Ding, L., Jing, H., Wang, T., Li, J., and Liu, G. (2010a). Regulation of root growth in Lactuca sativa L. seedlings by the ent-kaurane diterpenoid Epipodinosis. J. Plant Growth Regul. 29, 419–427. doi: 10.1007/s00344-010-9154-z

Donà, M., Ventura, L., Balestrazzi, A., Buttalava, A., Carbonera, D., Confolani, M., et al. (2014). Dose-dependent reactive species accumulation and preferential double-strand breaks repair are featured in the γ-ray response in Medicago truncatula cells. Plant Mol. Biol. Rep. 32, 129–141. doi: 10.1007/s11105-013-0635-7

Donà, M., Ventura, L., Macovei, A., Confolani, M., Savio, M., Giovannini, A., et al. (2013). Gamma irradiation with different dose rates induces different DNA damages responses in Petunia x hybrid cells. J. Plant Physiol. 170, 780–787. doi: 10.1016/j.jplph.2013.01.010

Endo, M., Ishikawa, Y., Osakabe, K., Nakayama, S., Kaya, H., Araki, T., et al. (2006). Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants. EMBO J. 25, 5579–5590. doi: 10.1038/sj.emboj.7601434

Endo, M., Nakayama, S., Umeda-Hara, C., Ohtsuki, N., Saika, H., Umeda, M., et al. (2012). CDK8RK2 is involved in mitosis and DNA damages response in rice. Plant J. 69, 967–977. doi: 10.1111/j.1365-313X.2011.04847.x

Frontiers in Genetics | www.frontiersin.org 15 June 2015 | Volume 6 | Article 216
Koppen, G., and Verscheure, L. (1996). The alkaline comet test on plant cells: a new genotoxicity test for DNA strand breaks in Vicia faba root cells. *Mutat. Res.* 360, 193–200. doi:10.1016/S0165-1161(96)90017-5

Koppen, G., and Verscheure, L. (2001). The alkaline single-cell gel electrophoresis/comet assay: a way to study DNA repair in radicle cells of germinating *Vicia faba*. *Folia Biol.* 47, 50–54.

Kozak, J., West, C. E., White, C., da Costa-Nunes, J. A., and Angelis, K. J. (2009). Rapid repair of DNA double strand breaks in *Arabidopsis thaliana* is dependent on proteins involved in chromosome structure maintenance. *DNA Repair* 8, 413–419. doi:10.1016/j.dnarep.2008.11.012

Kumar, A., Prasad, M. N., Achary, M. V., and Panda, B. B. (2013). Elucidation of lead-induced oxidative stress in *Talinum triangulare* roots by analysis of antioxidant responses and DNA damage at cellular level. *Environ. Sci. Pollut. Res. Int.* 20, 4551–4561. doi:10.1007/s11356-012-1354-6

Kwasniewska, J., Grabowska, M., Kwasniowski, M., and Kolano, B. (2012). Comet-FISH with rDNA probes for the analysis of mutagen-induced DNA damages in plant cells. *Environ. Mol. Mutagen.* 53, 369–375. doi:10.1002/em.21699

Kwon, Y., Abe, K., Endo, M., Osakabe, K., Namie Ohtsuki, N., Ayako Nishizawa-Yoko, A., et al. (2013). DNA replication arrest leads to enhanced homologous recombination and cell death in meristems of rice OsRecQ4 mutants. *RMC Plant Biol.* 13:62. doi:10.1186/1471-2229-13-62

Liman, R. (2013). Genotoxic effects of Bismuth (III) oxide nanoparticles by Allium and Comet assay. *Chemosphere* 93, 269–273. doi:10.1016/j.chemosphere.2013.04.076

Liman, R., Ciùçeri, I. H., and Özürkök, N. S. (2015). Determination of genotoxic effects of Imazethapyr herbicide in *Allium cepa* root cells by mitotic activity, chromosome aberration, and comet assay. *Pest. Biochem. Physiol.* 118, 38–42. doi:10.1016/j.psbp.2014.11.007

Liman, R., Ciùçeri, I. H., Akyıl, D., Eren, Y., and Konuk, M. (2011). Determination of genotoxicity of fenamnosulf by allium and comet tests. *Pest. Biochem. Physiol.* 99, 61–64. doi:10.1016/j.pestbp.2010.10.006

Lin, A., Zhang, X., Chen, C., and Cao, Q. (2007). Oxidative stress and DNA damages induced by cadmium accumulation. *J. Environ. Sci.* 19, 596–620. doi:10.1016/S1001-0742(07)60099-0

Lin, A., Zhang, X., Zhu, G., and Zhao, F. (2008). Arsenate-induced toxicity: effects on antioxidant enzymes and DNA damages in *Vicia faba*. *Environ. Toxicol. Chem.* 27, 413–419. doi:10.1897/07-266R.1

Liu, W., Zhu, L., Wang, J., Wang, J. H., Xie, H., and Song, Y. (2009). Assessment of the genotoxicity of Endosulfan in Earthworm and White Clover Plants Using the Comet Assay. *Arch. Environ. Contam. Toxicol.* 56, 742–746. doi:10.1007/s00244-009-9309-8

Love, A., Tandon, R., Banerjee, R., and Babu, C. (2009). Comparative study on elemental composition and DNA damages in leaves of a weed plant species, *Cassia occidentalis*, growing wild on weathered fly ash and soil. *Ecotoxicology and Environment Toxicology* 79, 801. doi:10.1080/09644029.2009.93225

Macovei, A., Garg, B., Raikwar, S., Balestrazzi, A., Carbonera, D., Buttafava, A., et al. (2012). Copper-induced oxidative damages, antioxidant response and genotoxicity in *Lycopersicon esculentum* Mill and *Cucumis sativus* L. *Plant Cell Rep.* 40, 1713–1721. doi:10.1007/s00299-011-1079-x

Pakrashi, S., Jain, N., Dalai, S., Jayakumar, J., Chandrasekaran, P., Raichur, A., et al. (2014). *In vivo* genotoxicity assessment of titanium dioxide nanoparticles by *Allium cepa* root tip assay at high exposure concentrations. *PLoS ONE* 9.e89828. doi:10.1371/journal.pone.0087789

Panagos, P., Liedekerke, M., Yigini, Y., and Montanarella, L. (2013). Contaminated sites in Europe: review of the current situation based on data collected through a European network. *J. Environ. Public Health* 2013:158764. doi:10.1155/2013/158764

Panda, B. B., and Achary, V. M. (2014). Mitogen-activated protein kinase signal transduction and DNA repair network are involved in aluminium-induced DNA damage and adaptive response in root cells of *Allium cepa* L. *Front. Plant Sci.* 5:236. doi:10.3389/fpls.2014.00256

Paparella, S., Tava, A., Avato, P., Biazz, E., Macovei, A., Biggiogera, M., et al. (2015). Cell wall integrity, genotoxic injury and PCD dynamics in alfalfa saponin-treated white pepper cells highlight a complex link between molecular structure and activity. *Physcochemistry* 111, 114–123. doi:10.1016/j.phytochem.2015.01.008

Patnaik, A. R., Achary, V. M. M., and Panda, B. B. (2013). Chromium (VI)-induced hromesis and genotoxicity are mediated through oxidative stress in root cells of *Allium cepa* L. *Plant Growth Regul.* 71, 157–170. doi:10.1007/s10725-013-9816-5

Petriccione, M., and Chiniglia, C. (2012). Comet assay to assess the genotoxicity of Persian walnut (*Juglans regia*) hulls with statistical evaluation. *Bull. Environ. Contam. Toxicol.* 89, 166–71. doi:10.1007/s00128-012-0637-4

Polli, P., de Mello, M. A., Buschini, A., Castro, V., Restivo, F., Rossi, C., et al. (2003). Evaluation of the genotoxicity induced by the fungus *Penicillium* in mammalian and plant cells by use of the single-cell gel electrophoresis assay. *Mutat. Res.* 540, 57–66. doi:10.1016/S1383-5718(03)00165-7

Pourrut, B., Jean, S., Silvestre, J., and Pinelli, E. (2011b). Lead-induced DNA damages in *Vicia faba* root cells: potential involvement of oxidative stress. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 726, 123–128. doi:10.1016/j.mrgentox.2011.09.001

Pourrut, B., Pinelli, E., Celié Mendiola, V., Silvestre, J., and Douay, F. (2015). Recommendations for increasing alkaline comet assay reliability in plants. *Mutagenesis* 30, 37–43. doi:10.1093/mutage/geu075

Pourrut, B., Shahid, M., Dumat, C., Winton, P., and Pinelli, E. (2011a). Lead-induced toxicity, and detoxification in plants. *Rev. Environ. Contam. Toxicol.* 213, 113–136. doi:10.1007/978-1-4419-9860-6_4
Procházková, D., Wilhelmová, N., Pavlíková, D., Sázková, J., and Gichner, T. (2013). Zinc induces DNA damages in tobacco roots. *Biuletyn Plantanum* 57, 783–787. doi: 10.24425/101533-013-0345-x

Qin, R., Wang, C., Chen, D., Björn, L. O., and Li, S. (2015). Copper-induced root growth inhibition of *Allium cepa* var. *agrotorum* involves disturbances in lipid, protein, RNA and DNA damage. *Environ. Toxicol.* 34, 1045–1105. doi: 10.1002/etc.2884

Radić, S., Cvjetko, P., Glavas, K., Roje, V., Pevalek-Kozlina, B., and Pavlica, M. (2009). Oxidative stress and DNA damages in broad bean (*Vicia faba L.*) seedlings induced by thallium. *Environ. Toxicol. Chem.* 28, 189–196. doi: 10.1897/08-188.1

Radić, S., Gregorović, G., Štipaničev, D., Cvjetko, P., Šrūta, M., Vujèvæ, Ž., et al. (2013). Assessment of surface water in the vicinity of fertilizer factory using fish and plants. *Ecotoxicol. Environ. Saf.* 96, 32–40. doi: 10.1016/j.ecoenv.2013.06.023

Radić, Š., Štipanicev, D., Cvjetko, P., Mikeliæ, I. L., Marjanoviæ, M., Rajaie, M. M., et al. (2010). Ecotoxicological assessment of industrial effluent using duckweed (*Lemna minor L.*) as a test organism. *Ecotoxicol. 19*, 216–222. doi: 10.1016/j.tox.2009.04-008-0

Rodríguez, E. (2011). Genotoxicity and Cytotoxicity of Cr(VI) and Pb2+. In *Pismum sativum*, PhD Thesis, University Aveiro (English vers.). Available online at: https://riia.ua.pt/bitstream/10773/7402/1/Thesi%20Definitiva%20EJR_imprimir.pdf

Rodríguez, E., Azavedo, R., Fernandez, P., and Santos, C. (2011). Cr(VI) induces DNA damages, cell cycle arrest and polyploidification: flow-cytometric and comet assay study in *Pismum sativum*. *Chem. Res. Toxicol.* 24, 1040–1047. doi: 10.1021/tx2001465

Rounds, M. A., and Larsen, P. B. (2008). Aluminium-dependent root-growth inhibition in *Arabidopsis* results from AtATR-regulated cell-cycle arrest. *Front. Plant Biol.* 9, 711–716. doi: 10.1016/j.plaphy.2010.06.023

Sakamoto, T., Inui, Y., Uraguchi, S., Yoshizumi, T., Matsunaga, S., Mastui, R., et al. (2013). Assessment of surface water in the vicinity of fertilizer factory using fish and plants. *Ecotoxicol. Environ. Saf.* 96, 32–40. doi: 10.1016/j.ecoenv.2013.06.023

Santos, A. R., Miguel, A. S., Macovei, A., Maycock, C., Balestrazzi, A., Oliva, A., and Gichner, T. (2013). Single Cell Gel Electrophoresis (Comet) assay with plants: research on DNA repair and ecogenotoxicity testing. *Chemosphere* 92, 1–9. doi: 10.1016/j.chemosphere.2013.03.006

Veprík, F., Konop, G., Schaeck, B., and Verschaeve, L. (2008). Automated detection of irradiated food with the comet assay. *Radiat. Prot. Dosimetry* 128, 421–426. doi: 10.1093/rpd/ncm433

Wang, Y., Xiao, R., Hailiang Wang, H., Zhiaoh Cheng, Z., Li, W., Zhu, G., et al. (2014). The *Arabidopsis* RADS1 paralogs RADS1B, RADS1D and XRCC2 play partially redundant roles in somatic DNA repair and gene regulation. *New Phytol.* 201, 292–304. doi: 10.1111/nph.12498

Waterworth, W. M., Kozak, J., Prosvit, C. M., Bray, C. M., Angelis, K. J., and West, C. E. (2009). DNA ligase 1 deficient plants display severe growth defects and delayed repair of both DNA single and double strand breaks. *MRC Plant Biol.* 9:79. doi: 10.1105/tpc.111.086314

Yildiz, M., Ciğerci, I. H., Konuk, M., Fidan, A. F., and Terzi, H. (2009). Determination of genotoxic effects of copper sulphate and cobalt chloride in *Allium cepa* root cells by chromosome aberration and comet assays. *Chemosphere* 75, 934–938. doi: 10.1016/j.chemosphere.2009.01.023

Zeng, X. Q., Chow, W. S., Su, L. J., Peng, X. X., and Peng, C. L. (2010). Protective effect of supplemental anthocyanins on *Arabidopsis* leaves under high light. *Physiol Plant.* 138, 215–225. doi: 10.1111/j.1399-3054.2009.01316.x

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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