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Review

Comet Assay: Multifaceted Options for Studies of Plant Stress Response

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Abstract: Contrarily to chronic stresses, acute (i.e., fast and dramatic) changes in environmental factors like temperature, radiation, concentration of toxic substances, or pathogen attack often lead to DNA damage. Some of the stress factors are genotoxic, i.e., they damage the DNA via physical interactions or via interference with DNA replication/repair machinery. However, cytotoxic factors, i.e., those that do not directly damage the DNA, can lead to secondary genotoxic effects either via the induction of the production of reactive oxygen, carbon, or nitrogen species, or via the activation of programmed cell death and related endonucleases. The extent of this damage, as well as the ability of the cell to repair it, represent a significant part of plant stress responses. Information about DNA damage is important for physiological studies as it helps to understand the complex adaptive responses of plants and even to predict the outcome of the plant's exposure to acute stress. Single cell gel electrophoresis (Comet assay) provides a convenient and relatively inexpensive tool to evaluate DNA strand breaks in the different organs of higher plants, as well as in unicellular algae. Comet assays are widely used in ecotoxicology and biomonitoring applications; however, they are still relatively rarely used in physiological studies. In this review, we provide an overview of the basic principles and of useful variations of the protocols of Comet assays, as well as of their use in plant studies, in order to encourage plant physiologists to include this tool in the analysis of plant stress responses.

Keywords: DNA damage; plant stress response; detection of DNA breakage; neutral and alkaline Comet assay

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1. Introduction

The maintenance of genome integrity is crucial for unicellular and multicellular plants to reach their full lifespan. The stability of DNA is required for proper growth and development, as well as for the faithful transmission of genetic material from one generation to the next. Because of their sessile nature, plants are constantly exposed to unfavorable conditions and cope with numerous DNA damaging factors, both endogenous (spontaneous, pre-programmed, or metabolically derived) and exogenous (e.g., atmospheric radiation, heat, desiccation, allelochemicals, and pollutants). Hundreds to thousands of DNA lesions are generated daily in each cell via different threats [1]. Breaks in a single strand or in both strands of the DNA represent a danger for the existence of the plant cell. However, almost all DNA lesions are rapidly and efficiently fixed through cellular DNA repair mechanisms.

Both the regulated destruction of DNA and the undesired events disturbing DNA integrity are associated with different aspects of plant life. They can occur during developmental events such as cell differentiation or the establishment and release of seed dormancy, and during stress responses induced, e.g., by pathogens, allelochemicals, or various abiotic factors. In all cases, genotoxic effects [2] can lead to short-term reversible genome damage or to the irreversible dismantling of the nuclear DNA in the course of the execution of programmed cell death (PCD).

For almost 40 years, single cell gel electrophoresis (SCGE), also known as the Comet assay, the single cell gel assay (SCG), or microgel electrophoresis (MGE), has remained one of the main cytogenetic tools for investigations of DNA lesions (strand breaks) and repair pathways in eukaryotic cells. SCGE was proposed in 1984 as a method for the detection of radiation-induced DNA breaks and was initially restricted to animal/mammalian systems [3]. Comet assays combine agarose electrophoresis methodology with fluorescence microscopy in order to observe and quantify DNA strand breakage at the level of single cells. The Comet technique was based on the following principles: Cells with damaged DNA exhibit increased migration of the chromosomal DNA from the nucleus in an electric field. The DNA migration pattern has a typical 'comet' shape, consisting of a head and a tail. The bulk DNA, also called the nucleoid ("the head"), moves from the cathode to the anode during electrophoresis more slowly than the short, broken DNA fragments ("the tail"), and the DNA then resembles a comet moving with the tail forward. Nowadays, this method is applicable for the detection of many DNA defects and is not limited to DNA strand break analysis.

Koppen and Verschaeve (1996) were the first researchers who adapted the Comet assay for the analysis of genotoxic effects in plants. *Vicia faba* roots were treated with seven mutagenic agents, and the isolated nuclei were evaluated for the extent of DNA migration [4]. From this experiment, it became clear that the Comet assay is well suited for applications in plants, and that the sensitivity of the Comet test is comparable to, or even higher than, the traditional chromosome aberration test or micronucleus test [5,6], as reviewed in [7]. By now, Comet assays have been applied to plants exposed to different adverse conditions, and described in a number of reviews and protocols [8–14]. The adaptation of the method to plants has led to a burst in research in the field of ecotoxicology, while highly valuable results were obtained also in phytopathology, embryology, and plant cell biology. The protocol of the Comet assay has been revised many times and modified for different objects and tasks, with ever-increasing reliability and reproducibility [14–17].

Importantly, it is not only genotoxic stress factors like UV radiation that lead to DNA damage; often, stress factors that are known as purely cytotoxic, like low temperature, induce the production of reactive oxygen, carbon, or nitrogen species (ROS, RCS, or RNS) that can damage the DNA, or activate endogenous cellular mechanisms that introduce DNA breaks, thus acting as "secondary genotoxic" factors [18–23]. The aim of this review is, therefore, to analyze the limitations and advantages of the Comet assay for various plant systems with special attention paid to stress tolerance research, in order to encourage plant physiologists to include this tool in their studies of plant responses to acute environmental changes that affect plant genome integrity.

2. Causes, Consequences, and Repair of DNA Lesions

DNA damage in plants can be caused by environmental agents or can arise from endogenous sources. Environmental genotoxins include factors such as ionizing radiation, UV light, heavy metals, and an excess of aluminum or boron, as well as natural toxins, for instance antibiotics of the bleomycin family [24–33]; there are also a number of chemical mutagens (reviewed in [34–36]). Endogenous factors leading to DNA damage include reactive oxygen, nitrogen, and carbon species (ROS, RNS, and RCS); the spontaneous or enzymatic release of DNA bases, leading to the formation of apyrimidine/apurine (AP) sites; errors in DNA replication and recombination; and the activity of plant DNases (reviewed in [1,37]).

DNA lesions include deletions of bases, the formation of pyrimidine dimers, cross-links (DNA–DNA cross-links and DNA–protein cross-links), single- and double-strand breaks (SSBs and DSBs, respectively), and base modifications such as alkylation or oxidation (reviewed in [1,36,38,39]). SSBs are produced through direct DNA oxidation via hydroxyl radicals or during transposition events. They are common products of DNA damage and are normally repaired, although their increased generation is widely accepted as a major source of inheritable mutations [40]. DSBs are the most cytotoxic DNA lesions that can

be caused by ROS (mainly via hydroxyl radicals generated during the decomposition of hydroperoxides in Fenton-like reactions), ionizing radiation (normally acting via a hydroxyl radical), or endonucleases; DSBs also result from stalled DNA replication or DNA transposition events [41–43]. The incorrect repair of DSBs can lead to the accumulation of mutations, chromosomal rearrangements, the appearance of aneuploid daughter cells, or cell death [44]. High levels of DNA damage can induce programmed cell death (PCD) in plant meristems [45].

An important endogenous factor leading to DNA damage is the enzymatic hydrolysis of nuclear DNA during senescence and PCD [46–48]. The hydrolysis of genomic DNA is performed via two major classes of S1-like nucleases, Zn^{2+}-dependent endonucleases and Ca^{2+}-dependent endonucleases [47]. Ca^{2+}-dependent nucleases act on double-stranded DNA (dsDNA) under neutral and optimal pH conditions; the activity of these nucleases increases temporarily at the beginning of PCD [49]. Contrarily, Zn^{2+}-dependent nucleases mainly act on single-stranded DNA (ssDNA) and RNA under acidic and optimal pH conditions and become activated at later stages of PCD [50]. Zn^{2+}-dependent nucleases are supposed to be released from the plastid or vacuole and to attack a large number of DNA fragments, completely degrading them [50]. Recently, it was shown that the Zn^{2+}-dependent nuclease CgENDO1 plays a direct role in the late degradation of the nuclear DNA in the process of PCD during secretory cavity in *Citrus grandis* 'Tomentosa' fruits [51].

In eukaryotes, DNA damage is sensed and leads to the activation of complex damage response pathways, collectively termed DDR (DNA damage response) which, depending on the severity and type of the damage, can activate processes such as cell cycle arrest, repair networks, or PCD (reviewed in [1,36,38,39,52]). The key sensors of DNA damage that elicit DDR in eukaryotes including plants are the ATAXIA-TELANGIECTASIA MUTATED (ATM) and ATM- and RAD3-related (ATR) protein kinases [53]. ATM is activated by DSBs, while ATR is activated by SSBs and by stalled replication forks. When activated, these kinases phosphorylate the transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), which represents a master switch for the genes involved in DNA repair and cell cycle arrest [54]. In plants, mechanisms of SSB repair include base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR), while DSBs are repaired via non-homologous end joining (NHEJ) and homologous recombination (HR) (reviewed in [1]).

The influence of genotoxic and cytotoxic stress factors that act on cell DNA and the possible outcomes are schematically shown in Figure 1.

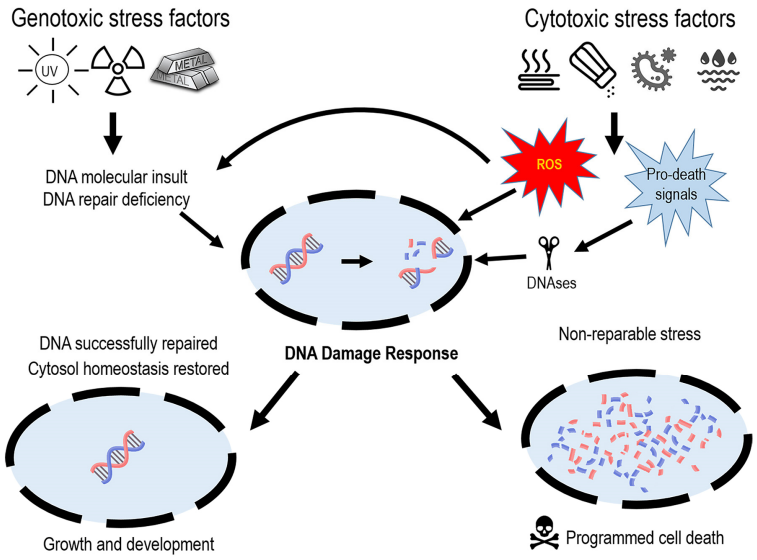

Figure 1. Both genotoxic and cytotoxic stress factors influence the DNA integrity in plant cells, rendering the evaluation of DNA damage an important part of the characterization of plant stress responses.

Comet assays serve as a powerful tool for the investigation of DNA repair [14,55]. A study of *Vicia faba* roots exposed to X-rays (2–50 Gy) by Koppen and Angelis (1998), showed that the repair of DSBs occurred in two phases: the first rapid stage with ca. 50% of breaks repaired within less than 20 min included NHEJ, while the second, slow stage required replication [24,56]. The kinetics of DNA repair in nuclei isolated from mature *Nicotiana tabacum* leaves after exposure to the alkylating agents ethyl methanesulfonate (EMS) and N-ethyl-N-nitrosourea (ENU) or to γ-radiation was studied as a function of time [57]. Comet assays showed that while the γ-induced DNA lesions were rapidly repaired, those induced by alkylating agents required much more time [57]. In another study, the role of long non-coding RNAs (lncRNAs) in DNA repair in plants was confirmed; Comet assays showed that mutants of *Arabidopsis thaliana* lacking three lncRNAs whose expression was upregulated by DNA damage experienced a drop in the DNA repair capacity [58].

3. Methods for Precise Detection of Genomic DNA Breakage

The integrity of nuclear DNA, i.e., the level of its non-enzymatic degradation and/or enzymatic cleavage, can be assessed via (1) the electrophoresis of extracted genomic DNA; (2) a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay; and (3) nuclear dispersion assays at the single cell level including the DNA diffusion test, the alkaline-halo assay (AHA), the fast halo assay (FHA), and the Comet assay with various modifications [59]. In the latter group of assays, the separation of DNA fragments can be performed either chemically, with radial diffusion of DNA fragments in alkaline milieu (DNA diffusion and halo assays) or electrically, with the linear migration of DNA fragments in an electric field (Comet assay). Altogether, nuclear dispersion assays show a relative simplicity, a high sensitivity, and require small numbers of cells per sample as compared with the electrophoresis of extracted genomic DNA.

The electrophoresis of extracted genomic DNA in 2% agarose gels with subsequent staining with DNA-binding dyes is a simple, low-cost, and efficient tool to resolve DNA fragmentation [60]. Degradation of nuclear DNA during PCD occurs gradually in two phases [50]. First, DNA is cleaved into large fragments of about 50 kb and 300 kb, corresponding to chromatin loops of ca. 50 kb and folds of six loops each forming 300 kb rosette structures, respectively. Second, these large fragments are further split by Ca^{2+}-dependent endonucleases that cleave at the linker sites between nucleosomes, producing DNA fragments of 180–200 bp in length [61]. Finally, random non-specific endo- and exonucleolytic cleavage leads to the formation of low-molecular-weight oligonucleotides and mononucleotides [62–64]. Altogether, about 30 nucleases are involved in DNA degradation in plants during PCD [64]. The visualization of the obtained DNA fragments via electrophoresis is called a "DNA ladder" [65]. The "DNA ladder" was observed during developmentally regulated PCD that takes place, for instance, during the degradation of the maternal tissue (nucellus) at early stages of wheat grain development [66], as well as during regulated stress-induced PCD, e.g., in the roots of six agronomic plants exposed to aluminum (maize, wheat, triticale, rye, barley, and oat; [67]), in wilting petals of *Antirrhinum majus*, *Argyranthemum frutescens*, and *Petunia hybrida* [68], or in wheat root cells during waterlogging [69]. The disadvantages of this method are the requirement of about 100–150 mg of fresh weight tissue for total DNA extraction and the restriction of the analysis to the level of tissue, not to single cells or nuclei. Furthermore, as DNA is isolated from thousands of cells, the results cannot be used for the detection of, e.g., low levels of apoptosis in some cells [70].

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay is an approach for the in situ labeling of damaged DNA in fixed plant material. TUNEL assays rely on the use of terminal deoxynucleotidyl transferase (TdT), an enzyme that attaches nucleotide triphosphates, tagged with a fluorochrome or another marker (X-dNTP), to the 3′-hydroxyl termini of DNA double strand breaks [71]. In parallel to TUNEL, the nuclei are stained with a second DNA marker, e.g., 4′,6-Diamidino-2-phenylindole

(DAPI), to enable the calculation of the percentage of damaged nuclei. The TUNEL assay was originally developed by Gorczyca et al. (1992) [72] and has been widely used for PCD investigations in plants since the study by O'Brien (1997) [73] (for recent analyses see [74–77]). Apoptotic-like DNA fragmentation can be estimated both on the basis of DNA laddering, and on the TUNEL assay. TUNEL assays provide an estimation of the extent of double DNA strand breaks but cannot determine the size of the cleaved products, as the localization of nuclear DNA fragmentation is performed in situ in fixed cells and tissues. The resolution of TUNEL assays allows for the detection of DNA breaks in a single nucleus with the possible analysis of specific localization within it; the assay is fast and relatively easy to carry out (reviewed in [78]).

The DNA diffusion assay was originally developed by Singh [70] as a simple, sensitive, and rapid method for the estimation of apoptosis in human leukocytes, and was optimized for different types of plant cells/tissues by Macovei et al. [79]. The assay includes mixing cells with agarose and preparing a microgel on a microscopic slide; the embedded cells are then lysed with salt and detergents, and the DNA is finally visualized via staining with a sensitive fluorescent dye. The shape of haloes in spots and the spot structure itself, i.e., the presence and size of a dense central zone, the clarity of the outer boundary of DNA diffusion, and the homogeneity of rings around the center, are used for the identification of nucleoids (structures resembling nuclei but lacking most histones and other nuclear proteins, and consisting of supercoiled loop DNA attached to the nuclear matrix; [80]) obtained from either viable, apoptotic-like, or necrotic plant cells [77,79].

In the alkaline-halo assay (AHA), nuclear DNA is prepared and deproteinized in a similar manner to the Comet assay procedure, but the dispersion of broken single strands and their separation from intact DNA is achieved via incubation in a high salt alkaline solution, followed by a hypotonic alkaline solution, without electrophoresis. The separation occurs in the gradient of the solvent concentration, and the diffusion of the DNA fragments through agarose pores results after staining with a fluorescent dye in the formation of a circular "halo" surrounding the bright nuclear remnants of damaged cells [81]. The radius of the halo is proportional to the extent of DNA damage. The fast halo assay (FHA) developed in 2006 [82] represents a simplified version of the AHA. The simplification of the lysis, denaturation, and staining procedures, as compared with AHA, results in a significant reduction in the total processing time [10,83].

Single cell gel electrophoresis (Comet assay) enables the investigation of DNA damage at the level of individual cells/nuclei with a high sensitivity. There are two versions of the Comet assay: the neutral and the alkaline one (see below). The neutral version was the first to be applied to plant material in 1993 for the identification of irradiated food originally presented at a workshop of the Commission of the European Community [84,85]. A few years later, a series of papers on DNA damage during seed storage was published starting from 1996 (Koppen and Verschaeve, 1996) using the alkaline version applied to broad bean (*Vicia faba*) radicles [4].

One of the most promising modifications of the Comet assay is the supplementation with lesion-specific endonucleases that reveals DNA lesions other than strand breaks. Thus far, twelve enzymes from the DNA repair machinery of bacteria or humans have been applied to animal and plant cells along with the Comet assay. "Net enzyme-sensitive sites" in nucleoids embedded in agarose can be scored through comparing the control non-treated samples with samples after incubation with the enzymes inducing extra DNA breaks (or AP sites) (reviewed in [86]). Nucleobase oxidation can be detected with the bacterial endonuclease III (EndoIII), catalyzing the excision of oxidized pyrimidines, or with formamidopyrimidine-DNA glycosylase (Fpg) and human 8-oxoguanine DNA glycosylase 1 (hOGG1), which catalyze the excision of oxidized purines (reviewed in [14]). Apart from DNA nucleobase oxidation, Comet assays are also used for the evaluation of inter-strand cross-links, which form a very detrimental type of DNA damage, representing the covalent binding of two complementary DNA strands via cross-linking agents [87,88]. A Comet assay followed by fluorescence in situ hybridization (FISH) can be used for the

detection of specifically labeled ("probed") DNA sequences of interest in the damaged or undamaged part of the comet (tail or head, respectively), and also to study the distribution of DNA damage and repair in the complete genome (reviewed in [89]). The combination of a Comet assay with the methylation-dependent endonuclease McrBC (a GTP-dependent restriction endonuclease of *E. coli* K12, selectively targeting DNA-containing modified cytosine residues) permits the evaluation of the global DNA methylation levels in populations of individual cells and is a highly important tool for epigenetic studies [90].

A modified version of the Comet assay, the Comet-based in vitro DNA repair assay, which was recently developed for mammalian cells, enables the quantification of the activities of two repair pathways, base and nucleotide excision repair (BER and NER) [91]. Protein extracts to be assayed are added directly to agarose-embedded nucleoids obtained from cells exposed to different DNA-damaging agents. This modification has not been applied yet to plants.

The diversity of the modified protocols of the Comet assay is highlighted in a number of reviews [14,92–94]. Here, we will focus on its standard neutral and alkaline versions and their implications for studies of plant stress responses.

4. Comet Test: Basic Principles and Useful Variations

Proposed in 1984 by Ostling and Johanson [3], the neutral Comet assay method was described in detail by Olive and Banáth (2006) [87], and since then was only slightly changed in subsequent publications. In contrast, the alkaline version of the Comet assay was published by Singh in 1988 [95] and since then has been modified many times. In the neutral version, the DNA is not denatured; hence, only double-strand breaks are detected. In the alkaline version, the DNA is denatured, allowing the quantification of both single- and double-strand breaks. The recently published compendium of Comet assay protocols includes a series of consensus protocols that specify the application of the alkaline version of the Comet assay (the so-called "standard alkaline Comet assay") to a wide variety of cell types, species, and types of DNA damage, with special attention to the appropriate methods of isolation of cells from different specimens [14].

4.1. Basic Principles

A Comet assay protocol includes the following main steps: the preparation of a suspension of cells or nuclei; the preparation of slides covered with agarose (gel slides); the resuspension of cells or nuclei in low melting point agarose; the application of the agarized suspension to gel slides; lysis; alkaline denaturation or preincubation in neutral solutions, depending on the protocol; the electrophoresis at alkaline pH or at neutral pH conditions, depending on the protocol; fixation (combined with neutralization step if electrophoresis was performed in alkaline conditions); staining; and microscopic analysis [14]. In recent decades, a number of highly informative and comprehensive reviews devoted to the peculiarities of Comet procedures in algae and higher plants have been published [10–13,96]. Recommendations and even guidelines pinpoint the most reliable laboratory practices to promote the generation of reproducible data, comparable between individual laboratories and research groups [16,94]. Here we list the most important features of the various steps of the Comet protocols which can affect the results, and precautions to be taken while performing the assay.

The general requirements are the avoidance of direct light, the maintenance of constant temperature, and the suppression of DNA-disturbing enzyme activities. All operations should be performed in a dark room under very dim light (0.1–1 lux) and at ice-cold temperature conditions, to prevent the destruction of nuclei and any artificial DNA damage during the assay. This is required because after the digestion of chromatin proteins, DNA is considered to be sensitive to light, especially to ultraviolet waves [13,16]. The duration of the different steps strongly depends on the type of plant cells under investigation [14].

For the high-resolution visualization of DNA migration, the suspension of nuclei or single cells is necessary. The main difficulty in preparing the suspension is the rigid

cell wall of plant cells, requiring mechanical and/or enzymatic processing in specific buffers; protoplasts have been used only rarely [97–99]. For the extraction of the nuclei, mechanical destruction of the cell wall is generally preferred over enzymatic lysis. The time of extraction has to be adjusted for different plant species and tissues to prevent inadvertent DNA damage.

Remarkably, the freezing of plant cells or nuclei for later analysis, including their submersion in liquid N_2 and subsequent storage (at −80 °C), has been reported only rarely [4,13]. Usually, fresh plant material is cooled on ice and disintegrated with a razor or scalpel blade. Chopping is more efficient than slicing and results in a suspension of intact nuclei within no more than 1–2 min. In most reports, roots or cultured cells are used as a source for the isolation of nuclei, but photosynthetically active plant tissues have been used also [15,100–102].

The next step is the preparation of bilayer agarose gel slides with embedded isolated nuclei. An ice-cold nuclear suspension is mixed with 0.75–1.5% low melting point (LMP) agarose (usually in equal volumes) at 37–42 °C via pipetting gently up and down, while avoiding the introduction of air bubbles. It is critically important to maintain the temperature of LMP agarose; too high a temperature would lead to the denaturation of proteins and the formation of DNA–protein crosslinking during the mixing step. Two drops are placed on an agarose-precoated microscope slide adjusted to 37 °C, covered by two cover slips and allowed to solidify via transferring the slide to an ice-cold surface. The quick cooling of the nuclei-containing drops is very important for preventing the activation of the DNAses. Agarose-precoated slides can be prepared by immersing the slides vertically for 2 s in molten 1% regular agarose (dissolved in distilled water, at 70–90 °C) and wiping one side clean. The slides with the thin agarose layer are then left to air-dry on an even surface for at least 24 h. The recommended number of nuclei is approximately 100 per gel [14].

In different protocols, the LMP agarose percentage can vary from 0.75 to 1.5% (*w*/*v*) [14], with most laboratories using a final agarose concentration of 0.8–1%. The agarose density is the factor which can influence the size and form of the tail of the comet, and is to be taken into account during electrophoresis, as otherwise it can lead to hidden, large, or lost, small, migrating DNA fragments [14].

The lysis with high salt-containing detergents removes cellular and nuclear membranes, the cytoplasm, the nucleoplasm, and most of the DNA-interacting proteins including all histones, while maintaining the interactions of DNA loop domains with the nuclear matrix. The resulting nucleoids are then incubated in a preincubation solution (with an optionally defined composition to induce, or not, the DNA unwinding) and placed in a weak electrophoretic field either at pH 8.0–8.5 (neutral Comet assay) or at pH > 13 (alkaline Comet assay). The loops of negatively charged relaxed DNA and the DNA fragments start to migrate from the nucleoids enclosed in agarose and form an electrophoretic track that looks like a comet tail. The formation of the tail is strongly facilitated if breaks are present in the DNA.

One controversial issue in Comet protocols, which can potentially impact the reliability of the assay and affect results, is the pH optimum of the lysis buffer [103]. While the pH of the lysis buffer likely has a negligible effect on the detection of DNA strand breaks, precise lysis conditions are highly important for the use of Comet assays for the detection of base modifications, which are often unstable and sensitive to pH [103].

At the final step of the assay, slides are usually observed via fluorescence microscopy and scored visually (whereby the tail and head parameters are estimated on the basis of their morphology) or using image analysis software to measure the distribution of DNA between the heads and tails of comets either via a semi-automatic (i.e., the investigator selects comets for the measurement) or a fully automatized method. Visual scoring categorizes comets into different classes depending on their shape, which is related to the length of migration path and/or the proportion of the DNA in the tail. A recently developed description of comet shapes allows the application of a 5- or 9-class visual scoring system [104]. Visual scoring is as reliable as the image analysis of comets, and the values of visual scoring (i.e., arbitrary

units) correlate well with the results of image analysis (i.e., % Tail DNA) [104]. Image analysis software permits the data processing that provides a suitable description of the DNA damage in each sample. These are: (1) primary Comet assay descriptors such as the percentage of fluorescence in the comet tail, the tail length, and the tail moment (product of the tail length and the fraction of total DNA in the tail); (2) use of statistical tools to obtain a central estimate of the distribution of a primary comet assay descriptor; and (3) methods to transform the central estimate to counts of lesions per unaltered nucleotides or per base pair [105,106]. At least 50 'comets' per gel from two gels have been recommended to analyze for the proper estimation of individual single cell comet tracks [107]. The classical version of the assay uses the 1–2 gels/slide format, i.e., one to two drops of cells mixed with low melting point agarose are placed on an agarose-precoated microscope slide. The CometChip® is a disposable gel, attached to a glass slide and indexed in 96 macrowells, each of which contains ca. 400 micropores patterned on the agarose for capturing individual cells. Comparison of the 2 gels/slide format with the CometChip® format using the alkaline version of the Comet assay revealed that the commercial CometChip® technology is very well suited for an increase in Comet assay throughput. In addition to increasing the number of samples treated in one run by about three orders of magnitude, it reduces the overlapping of comets, while the nucleoids/comets are all in the same focal plane, thus facilitating the scoring procedure [108]. At present, the use of the CometChip® is still rather rare, probably due to the relatively high costs per chip, but this method has a great potential for the large-scale investigations of DNA damage in plants.

4.2. Calibration and Positive Controls

In spite of the simplicity and reliability of the Comet assay, the use of calibration and positive controls is recommended. For studies on root cells, the use of roots of hydroponically grown plants (e.g., *V. faba* or *A. cepa*) as a model, and H_2O_2 as a damaging agent are recommended for calibration; H_2O_2 induces fast DNA damage, while not producing hazardous liquid waste. When the assay is to be performed with leaves, calibration tests must use a mutagen [16]. The most frequently applied chemical DNA strand break inducers in plants are the alkylating agents ethyl methanesulfonate (EMS), methyl metanosulphonate (MMS), and Methylnitrosourea (MNU), and the radiomimetics bleomycin and zeocin [4,102,109–127]. MMS, EMS, and MNU are capable of inducing a variety of lesions including adducts, cross-links, and breaks in the DNA chain; they can modify DNA, adding alkyl groups in several positions of the DNA bases. Bleomycin and zeocin are members of the bleomycin family of antibiotics and radiomimetic agents, which induce a spectrum of DNA lesions similar to that of ionizing radiation, namely a mixture of single-strand and double-strand breaks and abasic sites [127–129]. The Comet assays should be designed to yield an unbiased estimation, i.e., an assay should include both treated and control samples. Scoring should be performed investigator-blinded, irrespective of whether visual scoring or image analysis is used [14,106].

The calibration of Comet assays by treatments with a range of doses of ionizing radiation (X-rays or gamma radiation), and the establishment of laboratory-specific calibration curves can allow for the comparison of data between laboratories; also, transformation of results into Gy equivalents or into absolute frequencies of lesions can permit the comparison of effects on cells treated with any agent [130,131].

Furthermore, there are several parameters that should be specified when describing the results of the assay to achieve reproducible results, as variations in Comet assay procedures could hamper the inter-laboratory comparison and interpretation of data. In 2020, the international team of Comet assay experts developed and published the set of recommendations for the alkaline assay and how to correctly describe the conditions of the Comet assay and the obtained results [106]. These "Minimum Information for Reporting on the Comet Assay" (MIRCA) recommendations distinguish between 'desirable' and 'essential' information concerning the method descriptions, which could be included in the

papers. They also include useful recommendations on each step of the Comet assay for high quality results to be obtained.

4.3. Different DNA Organization within Comets in Neutral and Alkaline Variants

In plant cells, the genome is organized at three major levels: (i) the DNA molecule, (ii) the chromatin (DNA complexed with proteins, forming nucleosomes consisting of approximately 200 bp of DNA wrapped around a histone octamer), and (iii) chromosomes (folded chromatin; reviewed in [132–134]). At the same time, the structural organization of DNA within Comet preparations remains not fully understood.

In the neutral Comet assay, nucleoids (structures resembling nuclei that are produced during the lysis of agarose-embedded nuclei) consist of supercoiled DNA loops attached to residual nuclear structures. The supercoiling is a result of the removal of the nucleosomes from topologically constrained chromatin loops. The residual structure, the "matrix", may represent a result of aggregation of some DNA-bound proteins in high salt conditions. The tails of 'neutral' comets are formed by loops of relaxed dsDNA sized between 50 and 300 kb ([80,135,136], reviewed in [137]), associated with the residual nuclear matrix. In the neutral comet assay, the rate of DNA exit from the nucleoids depends on the topological state of the DNA. The appearance of haloes around nucleoids in control conditions is attributed to the relaxation of supercoiled DNA. A recently performed analysis of the kinetics of electrophoretic migration in the standard neutral comet assay and in a Pulsed-Field Comet Assay on human lymphocytes allowed the authors to distinguish between the single loops inside comet tails [80,138–140]. The data showed that the loops that migrate rapidly during the standard neutral comet assay in the interval between 0 and 30 min of electrophoresis are located on the surface of the nucleoid (i.e., they are the peripheral loops of the nucleus) and are relatively small (up to ~25 kb). The loops, whose migration is observed at the later stages of electrophoresis (i.e., between 30 and 120 min of electrophoresis), represent the inner and larger loops of the nucleus (from ~30 to ~150 kb).

Contrarily, the formation of comet tails in the alkaline Comet assay is not related to chromatin looping. The alkaline treatment induces the detachment of the loops from the nuclear matrix, and the tail is formed via ssDNA fragments that are pulled out from the comet head through electric force. There, a nucleoid represents a coil of ssDNA molecules that are not attached to the matrix. ssDNA can be detected in both the tail and the head of alkaline comets; their tails are supposed to contain ssDNA that has coalesced into 'granules' appearing more or less randomly over the tail area. The breaks arise from the nicks introduced in two ways: (i) directly via DNA damaging agents (before sample preparation); and (ii) as a result of the conversion of apurinic/apyrimidinic (AP) sites into nicks during alkaline treatment [80,136,141].

As discussed above, in the alkaline Comet assay, both the unwinding of the DNA and electrophoresis occur under alkaline conditions at pH $\geq$ 13, designated as A/A conditions. The A/A version is most often performed via the incubation of the agarose gel slides with nuclei in a buffer containing 0.3 M NaOH and 1 mM Na_2-EDTA for 40 min, followed by electrophoresis in the same buffer. The neutral Comet assay can be performed in two ways: either as the A/N version where DNA denaturation occurs under alkaline conditions and is followed by electrophoresis at neutral pH, or as N/N version where both the pre-incubation and electrophoresis are performed under pH-neutral conditions, usually with TBE buffer containing 45 mM Tris-borate and 1 mM EDTA at pH 8.0–8.4 [114,142]. The neutral N/N Comet assay is used to identify DSBs, while the alkaline (A/A) Comet assay and A/N protocol of the neutral Comet assay permits the detection of both DSBs and SSBs. However, at least some alkali-labile AP sites are not susceptible to conversion into breaks in the neutral A/N Comet assay and thus cannot be detected, although this version tends to have lower background levels of comet tail intensity than the all-neutral N/N assay [142]. The majority of alkali-labile AP sites can be detected with the A/A protocol [114,142].

The alkaline Comet assay (A/A version) uncovers the total damage in DNA, including SSBs, DSBs, and the presence of alkali-labile sites, and therefore represents a

universal method for the estimation of the extent of DNA damage in response to any stress factor [12,87,142]. If, however, the goal is to differentiate between SSBs and DSBs, then the two most different variants of the Comet assay with regard to pH, alkaline (A/A version) and neutral (N/N version), should be used in parallel as described by Gu et al. [127]; the difference between the results can reveal the portion of SSBs in the total pool of damaged DNA, e.g., during the reprogramming of differentiated cells into stem cells in *Physcomitrella patens* under stress-induced DNA damage.

To study the stress factors leading to DNA breaks caused by enzymes, e.g., by endonucleases in the course of PCD, one would need to estimate only the accumulation of SSBs and of DBSs, while minimizing the contribution of the latent modifications of nucleotides with alkali-labile properties in the formation of comets. To this aim, comparison should be made between the neutral Comet assay protocols using preincubation at alkaline conditions (A/N version) and without it (N/N version), respectively. Plant endonucleases cleave ssDNA as well as dsDNA; the appearance of 'DNA laddering' due to prevailing DSBs at the beginning of PCD is caused by the presence of histones shielding single strands of non-denatured DNA from endonucleases, but the portion of SSBs increases with progressing DNA denaturation [64]. Thus, the effects of PCD-inducing stress factors such as salinity, pathogens, or heat on DNA integrity should be estimated using both protocols (A/N and N/N versions) of the neutral Comet assay.

The effects of complex stress factors (e.g., allelochemicals, flooding, or exposure to heavy metals) with both genotoxic and cytotoxic effects on DNA should be evaluated over time using all three versions (A/A, A/N and N/N) of the Comet assays, as they can provide information on the prevailing type of damage in the course of the stress response.

5. Applications of the Comet Assay in Plant Studies

5.1. Comet Assays in Plant Ecotoxicology and Biological Monitoring

The predominant application of Comet assays to studies of plants over the world is in tests of the mutagenic and genotoxic effects of pollutants. Comet assays have revolutionized the field of genetic ecotoxicology, or eco-genotoxicology (reviewed in [11,143,144]), as they provide a non-specific, sensitive, rapid, and low-cost tool for the detection of genetic damage in natural biota, and for the biomonitoring of toxicants both in aquatic and terrestrial ecosystems. Compared to assays based on animal studies, plant-based bioassays are easy to perform, while providing information on the induction of DNA strand breaks in somatic and germ cells that is relevant for the whole biota. Traditional plant bioassays include the detection of chromosomal aberrations or exchanges between sister chromatids; tests for point mutations with visible phenotype are also useful (e.g., chlorophyll mutations, waxy mutations, or embryo mutations of Arabidopsis; reviewed in [145]). In recent years, even more analyses are being performed on plants using Comet assays [11,143,144]. The advantages and limitations of Comet assays applied to animal models for eco-genotoxicology and biomonitoring have been discussed [146]; most of them are also valid for plants.

An example of the successful use of the Comet assay in a commercial application is in the estimation of drinking water quality, a vitally important parameter for human health and longevity. The genotoxicity of perfluoroalkylated and polyfluoroalkylated substances (PFASs), a group of man-made chemicals contaminating drinking water along the entire supply chain, to a Crustacean, an alga, a plant, a bacterium, and a human leukocyte culture was evaluated in a study by Alias [143]. The authors concluded that the Comet assay is a highly promising tool for use in water quality control services, in order to provide the necessary quality characteristics of raw water in critical situations [143].

The leaves of deciduous plants can be used as bioindicators of the DNA damage caused by various genotoxic factors such as radiation, chemicals, allelochemicals, heavy metals, nanoparticles, or complex contaminants originating from the atmosphere and soil. *Ligustrum vulgare* (common privet) has been used as a model to monitor the air pollution using Comet assays: analyses of leaves harvested at three locations with different levels of air pollution in Bosnia and Herzegovina, including two urban and one rural location,

showed that DNA damage measured as tail intensity depended on the sampling period, leaf position, and growth stage, and showed clear differences between urban and rural locations [147]. Furthermore, this model proved sensitive to seasonal variations in air pollution levels since DNA damage in *L. vulgare* leaves was proportional to the average concentration of particulate matter of a size ranging from 0.001 to 2.5 μm in diameter ($PM_{2.5}$) and depended on indoor vs. outdoor conditions [147].

Another expanding area of Comet assay applications is the evaluation of the genotoxic effects of newly developed herbicides. For example, cytotoxic and genotoxic effects of imazethapyr, an imidazolinone herbicide, were investigated using root meristem cells of *Allium cepa* [148]. The results indicated that imazethapyr exhibits cytotoxic activity and induces DNA damage in a dose-dependent manner. Comet assays were used to examine the cytotoxic and genotoxic effects of the herbicides penoxsulam, pinoxaden, and clopyralid on *A. cepa* roots. All three compounds showed a cytotoxic effect by reducing the root growth and mitotic index, and a genotoxic effect by increasing chromosome aberrations and DNA damage, as compared to control roots [124,125,149]. Furthermore, the genotoxic effects of two herbicides representing synthetic auxins, picloram and dicamba, on root meristems of *A. cepa* were evaluated utilizing Comet assays in roots grown either under conditions simulating tissue culture (i.e., aseptic conditions using Murashige and Skoog (MS) medium) or in bidistilled water [150]. Both herbicides induced a more severe stress and more pronounced DNA damage in the cells of roots grown under tissue culture conditions. This study confirmed the genotoxic effects of two growth regulators on plant cells [150].

5.2. Comet Assays in Plant Physiological Studies

The Comet assay has proved a valuable research tool and, as such, has been used in plant radiation biology, embryology, and studies on plant development and responses to abiotic and biotic stresses, as well as in experimental data modeling. In plant molecular studies, Comet assays are utilized to characterize the mutants impaired in nuclear DNA repair functions, in DNA damage sensing/signaling, and chromatin remodeling [14,151–155]. Furthermore, Comet assays can be applied to studies of DNA replication in chloroplasts. In chloroplasts, transcription–replication conflicts can lead to the formation of R-loops, temporary hybrids between template DNA and nascent mRNA, which can block replication fork progression and provide a major source of genomic instability. In a recent study, the Comet assay (neutral version) was used to characterize the mechanism involved in R-loop formation via assessing the chloroplast genome integrity in mutants [156].

Comet assays are also very useful for the complex characterization of instability induced in plant genomes by various stress factors [157]. Moreover, the alkaline Comet assay provides an efficient tool for differentiating between agents that represent sources of genotoxic and purely cytotoxic damage. For instance, Comet assays performed with human leukocytes showed that camptothecin, an inhibitor of topoisomerase I, or actinomycin D, an inhibitor of RNA synthesis, both provoked DNA strand breaks (i.e., comets were observed in the assays), while chemicals that do not bind DNA such as cordycepin, an mRNA synthesis inhibitor that blocks the elongation of the growing RNA chain, fluorodeoxyuridine, blocking the action of thymidilate synthetase, or puromycin, an inhibitor of protein synthesis, did not induce the formation of comets [158]. Below, we discuss how the Comet assay can be used for studies on plants exposed to stress factors causing, directly or indirectly, damage to DNA.

5.2.1. Use of the Comet Assay in Studies on Plants Exposed to Genotoxic Stress Factors

Common genotoxic stress factors include ionizing radiation such as gamma irradiation, X-rays, and short-waved UV-C, as well as exposure to radiomimetics, a large group of compounds acting directly on DNA-like alkylating agents and antibiotics of the bleomycin family, or drugs inhibiting DNA synthesis and repair like, e.g., antimetabolites [32,33].

Gamma rays are the most effective ionizing radiation used in agricultural programs, for instance, for the surface sterilization of agricultural products in order to increase their

conservation time via reducing pathogen propagation. The effects of γ-radiation on the nuclear DNA of *Vicia faba* seeds either without treatment or treated with ZnO nanoparticles were studied using flow cytometry, an alkaline Comet assay, and the random amplification of polymorphic DNA (RAPD)-PCR; all three assays confirmed that the treatment of the seeds with ZnO nanoparticles preserved their DNA integrity [159]. Furthermore, γ-radiation is an attractive alternative to the chemicals used against an obligate endoparasite, the root-knot nematode *Meloidogyne incognita* that can infect most vegetables, fruits, and ornamental plants all over the world [160]. Analysis of the effects of low doses of γ-radiation on the infectivity of *M. incognita*, growth of tomato and pepper host plants, and DNA breakage in leaf cells using neutral Comet assays showed that the treatment of seedlings with γ-irradiation is a promising technique for nematode control without any suppressive effects on the host plants [160].

The effects of seed pre-treatment with cold atmospheric-pressure air plasma on DNA and on the induction of a positive adaptive response in seedlings were studied in pea seedlings [122]. The DNA damage was analyzed using alkaline comet assays, and the induced adaptive response was tested using the toxic concentrations of a DNA double-strand break inducer, the glycopeptide antibiotic zeocin. At all exposure times studied, seed pre-treatment with plasma exerted a protective effect on seeds and subsequently led to a reduction in DNA damage in zeocin-treated pea seedlings, in comparison to seedlings germinated from control seeds without plasma treatment. These results not only confirmed that plasma can be safely used in agriculture for seed treatment, but also showed the existence of a plasma-induced adaptive response [122].

The exposure of plants to heavy metals can decrease the growth and yield of crops, and produce toxic, mutagenic, and carcinogenic effects in natural biological systems. Prolonged exposure to physiological concentrations of heavy metals, e.g., chromium, can inhibit the enzymes of DNA repair mechanisms and cause the formation of DNA adducts, leading to direct DNA damage like SSBs, DSBs, base modifications, and DNA–DNA/protein cross-linking, resulting in genotoxic effects [161]. Furthermore, the exposure of plants to heavy metals initiates ROS formation and other cytotoxic effects in the cytosol that may also lead to DNA damage [162–164]. Alkaline Comet assays were used to evaluate the extent of DNA damage in leaf and root cells of Indian mustard (*Brassica juncea*) and yellow lupin (*Lupins luteus*) caused by lead [27,165]. The accumulation of lead inside cells was proportional to the amount of DNA fragments migrating away from the tail of DNA comets, as well as to the degree of cell damage. Another study using neutral Comet assays showed that exogenously applied jasmonic acid decreased the DNA damage incurred by the lead treatment [166].

The DNA damage and chromatin degradation were evaluated using alkaline Comet assays and fluorescent immunolabeling following the exposure of the roots of *Allium cepa* and *Vicia faba* to the organophosphate insecticides fenthion and malathion and to two heavy metal salts, nickel nitrate and potassium dichromate [167]. Severe DNA damage in the cells of the roots treated with the highest doses of both stressors was associated with the induction of apoptosis-like programmed cell death [167].

Arsenic toxicity was evaluated using alkaline Comet assays [168,169]. In both the leaves and roots of *V. faba* and *Cucumis melo* grown hydroponically in liquid medium supplemented with disodium hydrogen arsenate, a loss of DNA integrity was shown. In another study, neutral Comet assays showed that vanadium treatment leads to DNA fragmentation in *Allium cepa* roots with the level of DSBs increasing depending of the VCl_3 dose, possibly indicating the induction of PCD [29].

A study comparing the effects of four heavy metals (cadmium, zinc, copper, and lead), the preservative sodium benzoate, and wastewater on DNA integrity was performed with the roots of *Allium cepa* using the neutral Comet assay [170]. The results showed that the DNA damage, depending on the comet tail length, was highest in the presence of cadmium and decreased in the presence of the other pollutants in the order cadmium > zinc > sodium benzoate > copper > lead > wastewater. Interestingly, the pretreatment of barley

seedlings with a non-toxic dose of cadmium chloride prior to exposure to MNU reduced the frequency of chromatid aberrations and the formation of micronuclei and aneuploid cells, as well as the amount of DNA in comet tails [113].

5.2.2. Use of the Comet Assay in Studies on Plants Exposed to Cytotoxic Stress

Salinity is one of the major abiotic stress factors and affects the productivity of agricultural crops worldwide. Both Na^+ and Cl^- ions cause a number of cytotoxic effects on cells and organelles [171,172]. Furthermore, an increase in ROS production and a decrease in cytosolic K^+ can lead to the induction of PCD; one of the important steps in this pathway is the activation of the endonucleases that introduce dsDNA breaks [173–175]. Below, we discuss several studies showing, on the basis of the Comet assay, that exposure to NaCl can be accompanied by damage to DNA; more examples are listed in Table 1.

Table 1. Some examples of the successful application of Comet assays to studies of plant stress responses.

Stress Factor	Plant Species/Organ	Version of the Comet Assay	Type of Data Processing	Reference
Predominantly genotoxic stress factors				
Heat	leaves of *Vicia faba*	neutral	visual assessment of comet shape	[176]
	leaves and roots of *Nicotiana tabacum* var. *xanthi*	alkaline	Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK	[8]
	leaves of *Oryza sativa*	alkaline	visual scoring (four classes of damaged DNA)	[177]
	Arabidopsis thaliana seedlings	neutral	Casp-1.2.3b2 software	[178]
	leaves of *Lycopersicon esculentum*	neutral	ImageJ version 1.54h	[179]
	leaves of *Vicia faba*	neutral	visual assessment of comet shape	[180]
	leaves of *Solanum tuberosum*	neutral	TriTek CometScore™ (version 1.5, Sumerduck, VA, USA)	[181]
Heavy metals	roots of *Brassica juncea*	alkaline	CASP software	[166]
	roots of *Brassica juncea*	alkaline	Carl Zeiss Axiovision software (Special Edition 64 Rel. 4.9.1)	[27]
	leaves of *Vitis vinifera*	alkaline	visual scoring (four classes of damaged DNA) followed by FISH	[28]
	roots of *Vicia faba* and *Allium cepa*	alkaline	Comet Assay IV (Perceptive Instruments)	[167]
	leaves of 30-day-old *Solanum lycopersicum*	alkaline	CASP software	[182]
	root tips of *Allium cepa*	neutral	Comet Assay Software (CASP-version 1.2.3b)	[29]
	root tips of *Lupinus luteus*	alkaline	Scion Image analysis system	[165]
	the roots and shoots of *Cucumis melo* seedlings	alkaline	visual scoring and ranking into classes 0–4 (non-damaged–maximally damaged)	[169]
High light + heat	leaves of *Oryza sativa*	neutral	CASP software	[123]
Pathogen	leaves of *Arabidopsis thaliana*	neutral	TriTek CometScore software (Tritek Co., Sumerduck, VA, USA)	[183]
	leaves of *Nicotiana tabacum* var. *xanthi*	alkaline	Komet v. 3.1, Kinetic Imaging, Liverpool, UK	[184]
	leaves of *Lycopersicon esculentum*	alkaline	Tritek Comet score version 1.5	[185]
	Arabidopsis thaliana seedlings	neutral	CometScore software (Tritek Co, Sumerduck, VA, USA)	[186]
Cytotoxic stress factors				
Salt stress	shoots and roots of *Vigna radiata* seedlings	alkaline	Komet 5.5 (Kinetic imaging, Andor Technology)	[187]
	leaves of *Hordeum vulgare* seedlings	neutral	CASP software (Comet Assay Software Project)	[188]
	leaves of *Brassica juncea*	alkaline	CASP software (Comet Assay Software Project)	[189]

Table 1. *Cont.*

Stress Factor	Plant Species/Organ	Version of the Comet Assay	Type of Data Processing	Reference
Salt stress	shoots of 8 d and 12 d old seedlings of *Oryza sativa*	neutral	CASP software (Comet Assay Software Project)	[190]
	root tip cells and 7 d old seedlings of *Hordeum vulgare*	alkaline	OpenComet v1.3.1 Software	[191]
	leaves of 32-day-old *Vicia faba*	alkaline	Komet 5 software (Liverpoo1, UK)	[192]
	roots of *Allium cepa*	alkaline	Carl Zeiss Axiovision software	[18]
	germinated seeds of *Solanum melongena*	neutral	CaspLab software	[193]
	protonema of *Physcomitrella patens*	neutral (with and without unwinding step before electrophoresis)	Comet module of LUCIA cytogenetics software suite (LIM, Praha, Czech Republic)	[19]
Flooding	root tips of *Zea mays* seedlings	alkaline	not indicated	[20]
Oxidative stress (Ozone; H_2O_2)	leaves of 70-day-old *Nicotiana tabacum*	alkaline	Comet assay III, version 3.0; Perceptive Instruments Ltd., UK	[21]
	leaf protoplasts of *Arbutus unedo* and *Populus maximowiczii Henry* × *berolinensis*	alkaline	Comet Assay III, Perceptive Instruments, UK	[99]
	leaves of 4-year-old *Ulmus glabra* seedlings	alkaline	Comet Assay III, Perceptive Instruments, UK	[194]
Allelochemicals	leaves of *Glycine max*	alkaline	Komet Version 3.1. Kinetic Imaging, Liverpool, UK	[195]
	root tips of *Lactuca sativa* seedlings	alkaline	CASP software	[22]
	roots of *Lactuca sativa* seedlings	alkaline	scoring with the classification of comets into 0 to 4 according to shape	[23]

Neutral Comet assays performed with the leaves of barley genotypes with different salt tolerance showed that after two weeks of plant growth in a hydroponic medium supplemented with 300 mM NaCl, DNA damage in leaf cells of a salt-sensitive cultivar significantly increased; this was accompanied by a rise in ROS production and a drop in the activities of antioxidant systems [188].

The alkaline version of the Comet assay was successfully used to show that pre-treatment of *Vigna radiata* (mungbean) seeds with sublethal doses of NaCl can alleviate the injurious effects of the later application of NaCl stress on roots and leaves of 7 d old seedlings [187]. As confirmed by Comet assays, an increase in DNA damage was caused by a NaCl-induced rise in ROS in a dose-dependent manner [187]. Alkaline Comet assays also revealed the cytoprotective role of the brassinosteroid 24-Epibrassinolide (EBL) in *Brassica juncea* (Indian mustard) under salinity stress. Exogenously applied EBL diminished the deleterious effect of 100 mM NaCl on DNA, as evidenced via a decrease in tail length and tail moment of the comets. The EBL-induced protective mechanisms were based on the prevention of the accumulation of ROS [189].

The role of DNA polymerase λ (*OsPolλ*) in DNA repair and in plant tolerance to salinity and drought stress was studied on three *Oryza sativa* cultivars [190]. The damage to genomic DNA was assessed using neutral Comet assays. Both *OsPolλ* gene expression and enzymatic activity were enhanced in response to these stresses, and higher DNA damage was associated with higher *OsPolλ* expression and enzyme activity.

Interestingly, pre-treatment with L-carnitine reduced the genotoxic effects of salinity, as shown by a study of seed germination and cell division in the root meristem cells of barley seedlings [191]. L-carnitine facilitates the transport of long chain fatty acids from the cytosol into the mitochondria and acts in lipid metabolism, presumably through the management of specific acyl-CoA pools [196]; the exogenous application of L-carnitine can enhance plant growth [197]. Alkaline Comet assays' results showed that exogenously applied L-carnitine alleviated the harmful effects of salt stress as it led to the increased mitotic activity of root meristem cells and reduced the levels of DNA damage.

Allelopathic compounds, also called allelochemicals, are biologically active secondary metabolites produced by 'donor' plants in order to suppress the growth of their competitors—'recipient' plants of the same or different species. In many studies of plant–

plant interactions, a reduction in DNA integrity was observed in recipient plants. This corresponds well to the fact that in many cases, allelochemicals induce ROS-mediated PCD in the recipient plants (e.g., [198]).

Narciclasine, an Amaryllidaceae alkaloid isolated from *Narcissus tazetta* bulbs, inhibits root growth due to cell cycle arrest, and causes the accumulation of ROS and an increase in DNA damage in the cells of lettuce roots [22]. A forest tree, *Eucalyptus globulus*, is able to produce allelochemicals, which accumulate in rhizosphere soil at high concentrations. When the fine powder of dried *Eucalyptus* leaves was added to a soil mixture for soybean, an increase in DNA damage was revealed through alkaline Comet assays, accompanied by an enhancement of the transcript levels of a legumain-like cysteine protease VPE3, indicating that the *Eucalyptus* leaf powder induced an apoptotic response to allelopathic stress [195].

The genus *Solanum* is the largest in the family Solanaceae, and *Solanum* species produce various allelochemicals, e.g., steroidal glycoalkaloids. The allelopathic effects of the hydroalcoholic extracts from two *Solanum* species, *S. muricatum* and *S. betaceum*, on lettuce were investigated; alkaline Comet assays indicated that both extracts induced DNA damage in leaf cells [23].

Strikingly, Comet assays have revealed a link between the plant immune response and DNA damage repair. Accumulation of DSBs in *Arabidopsis* was observed following infection by plant pathogens: a bacterium, a hemibiotrophic oomycete, and a necrotrophic fungus. Non-pathogenic *E. coli* did not produce this effect and thus served as negative control [183]. The genotoxicity of microbial infections could be attributed mainly to the effectors produced by pathogens and not to host-generated ROS [183]. Potato virus X (PVX) induced DNA damage in the nuclei of its host, tobacco (*Nicotiana tabacum* var. *xanthi*) [184]. Neutral Comet assays detected chronic genotoxic stress in an insertional mutant of *Arabidopsis* impaired in the nuclear DNA mismatch repair mechanism; the phenotype included the activation of DDR-associated genes and an increased resistance against *Pseudomonas syringae* [186]. Thus, the constitutive expression of DDR and DSB repair genes seems to contribute to an increased plant resistance to pathogens [186].

Heat stress is one of the major abiotic stress factors that induces heavy DNA damage in plants including nucleotide modifications, formation of SSBs and DSBs, and changes in chromatin architecture (reviewed in [178]); it also inhibits DNA repair mechanisms [199]. In higher eukaryotes, hyperthermia leads to the activation of highly conserved defensive mechanisms known as the heat stress response, in which the heat shock transcription factors (HSF) enhance the transcription of heat shock proteins' genes (*HSP*) via directly binding to the heat response elements in their promoters (reviewed in [178]). Recently, the high expression of osmotically responsive genes 1 (HOS1), an E3 ubiquitin ligase, was shown to play a role in the establishment of basal thermotolerance. As was shown by neutral Comet assays, HOS1 induced basal thermotolerance via the activation of DNA repair genes, acting as a transcriptional co-regulator [178]. Contrarily to basal thermotolerance, acquired thermotolerance depends on the heat stress memory that is often "encoded" by chromatin modifications [178]. Thus, under heat stress, DNA repair is mediated by the thermoresponsive HOS1-dependent pathway, in addition to the ATM-dependent pathway for repairing DSBs and, possibly, the ATR-dependent pathway for repairing SSBs.

Comet assays were used to monitor DNA damage in plant cells under heat stress in several more studies (see also Table 1). Experiments on *Vicia faba* leaves showed that DNA damage increased with increasing levels of heat stress, correlated with the in situ production of ROS, the number of dead cells, and the activity of proline synthesis [176]. Comet assays revealed the important role of the *temperature enhanced lesion spots 1* (*hes1*) mutation in rice in the activation of the heat stress response; the mutation led to ROS production, development of necrotic spots, and a high thermosensitivity of *hes1* plants [177].

Last but not least, the dual role of ROS physiology should be kept in mind. Some developmental changes involve a sharp increase in ROS production, which represents an intrinsic source of DNA damage in plants, and therefore has to be kept within a limit

called the "oxidative window". For instance, during the period of seed imbibition, cells need to maintain a fine balance between the production of ROS acting as oxidative signals promoting germination, and germination-delaying oxidative damage [200]. As shown in a study on *Medicago truncatula* seeds using alkaline Comet assays on radicles and 4-day-old seedlings, DNA repair is critical for the successful emergence of the radicle during the initial rehydration of the seeds, since DNA damage accumulates during maturational seed drying and storage due to poor repair capacities [201]. In another study, neutral Comet assays showed that the priming of the seeds of *Solanum melongena* with low NaCl concentrations enhanced the germination rate, leading to an increase in ROS levels but a decrease in DNA damage levels [193].

Visualizations of typical comets in nuclei suspensions obtained from the leaves of barley and onion that had been subjected either to the radiomimetic zeocin or to heat stress are shown in Figure 2. Times and voltage of electrophoresis were adjusted to obtain minimal DNA migration in the control group and, at the same time, maximal sensitivity to DNA damage caused by zeocin.

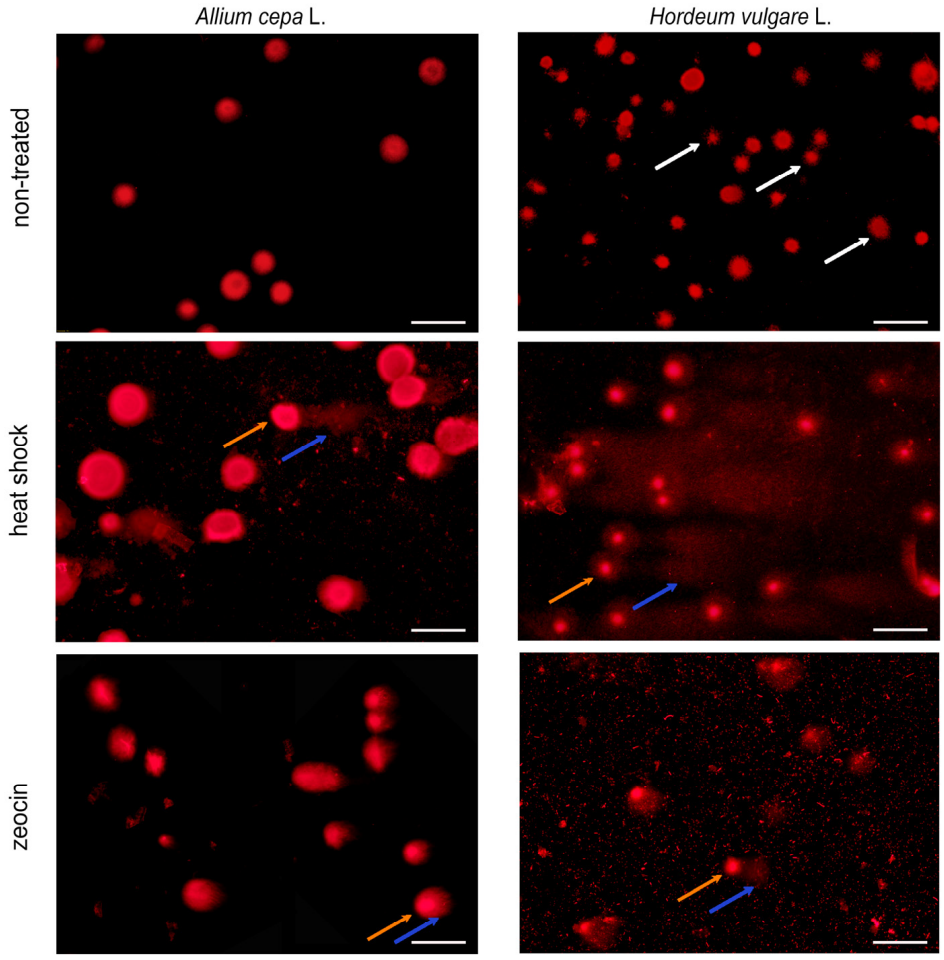

Figure 2. Neutral Comet assay analysis showing nuclei with comets in leaves of onion (*Allium cepa*) and barley (*Hordeum vulgare*) after DNA damage-inducing treatments. Nuclei were extracted from the leaves of onion or barley seedlings and were subjected to treatment by two stressors, heat shock (55 °C, 1 h) or the radiomimetic antibiotic zeocin (100 µg/mL, 24 h). Neutral comet assays (N/N protocol) were performed following the method of Menke et al. (2001) [114] with modifications; leaf nuclei were isolated according to Kubalová et al. (2021) [202] for barley and to Collins et al. (2023) for onion, with modifications [14]. For more information, see Supplementary Information File S1. Orange arrows point at the heads of comets, blue arrows at the tails of comets, and white arrows point at nuclei with slightly relaxed DNA. Scale bars: 100 µm.

Note that, although conditions and protocols of the Comet assay were identical, the procedures of the isolation of the nuclei were not (Supplementary Information File S1), and subtle differences can be seen between the two species, illustrating the necessity of the optimization of the whole method for every plant species and organ/tissue. While in non-treated control samples from both species, comets and debris of degraded nuclei were absent, heat shock-treated nuclei were losing their round shape, and "haloed" nuclei were observed where strand breaks had led to the release of supercoils and DNA relaxation, as well as comets with the accumulation of fragmented DNA in the comet tails. Zeocin-mediated DNA cleavage resulted in the formation of comets with relatively short tails of less than half of the total comet length. The high level of background debris in zeocin-treated barley samples is thought to be related to the strong disintegration of the nuclei. Note also that in barley control samples, some of the nuclei show protrusions indicative of the beginning of DNA relaxation; the reason for this might be the release of proteolytic and/or nucleolytic enzymes during the isolation of the nuclei, indicating that the procedure should be further optimized.

6. Conclusions and Outlook

Compared to other tests of DNA damage caused by genotoxic and cytotoxic stress factors, as well as by developmental ROS production, Comet assays show several advantages in (a) their high sensitivity enabling the detection of even low levels of DNA damage, (b) the requirement for only small numbers of cells per sample, and (c) flexibility, low costs, ease of application, and the relatively short time needed to complete a study. Furthermore, different variations of Comet assays allow refined studies of the types of DNA damage, as well as following DNA repair mechanisms induced by various stress factors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10020174/s1, Supplementary Information File S1: Protocol of the neutral Comet assay.

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