https://doi.org/10.31489/2025FEB3/128-137

UDC 576.3:575:577.21 Received: 22.05.2025 | Accepted: 15.07.2025 | Published online: 30 September 2025

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Comparative analysis of the COMET, FISH, and TUNEL methods for assessing DNA damage in plants under abiotic and biotic stresses

Research that investigates plant DNA damage caused by various stress factors represents an important area of modern molecular biology and genetics. In recent decades, there has been active development of methods that allow for detailed analysis of molecular responses in plants to abiotic and biotic stresses, significantly deepening our understanding of the mechanisms underlying their adaptation to adverse conditions. One of the key aspects of such studies is the assessment of damage to genetic material, which plays an important role in disrupting the normal functioning of plant cells and tissues. Special attention is paid to the combined effects of stress factors such as high fever and viral infections, such as Tobacco bushy stunt virus (TBSV) infection, which can significantly disrupt DNA integrity and normal cellular processes. This, in turn, can lead to changes in the activity of key genes, DNA repair, as well as effects on the physiological and morphological characteristics of plants. In this article, we examined three methods that are actively used to assess DNA damage under combined stress conditions: the COMET, TUNEL and FISH. These methods allow for a comprehensive analysis of DNA damage, as well as to investigate their relationship to physiological and cellular changes in plants exposed to viral and temperature stress. The purpose of this study is to explore the prospects of using the COMET, FISH, and TUNEL assay methods to assess the level of damage to plant DNA caused by abiotic and biotic stress. The research is aimed at analyzing their effectiveness, as well as identifying advantages and limitations when working with plant objects.

Keywords: *TBSV, Nicotianabenthamiana*, combined stress, DNA damage, oxidative stress, DNA repair, COMET assay, TUNEL assay, FISH hybridization.

Introduction

Plants, as immobile organisms, are constantly exposed to various abiotic and biotic stressors, such as high temperatures, drought, UV radiation, and pathogens, including viruses. One of the most serious consequences of stress is DNA damage, which can lead to mutations, genomic instability, and even cell death [1–4]. Scenarios of combined stress (e.g., heat plus viral infection) are especially impactful because the factors act synergistically to intensify oxidative damage, perturb replication and repair, and reprogram stress-responsive gene networks [5, 6]. Given the broad adoption of *Nicotiana benthamiana* as a model for plant–microbe and plant-virus interactions, and its recently improved reference genome, the system is well-suited to dissect stress-induced genome instability [7].

One of the widely studied viruses that have a significant effect on plants is Tobacco bushy stunt virus (TBSV), a virus with positive single-stranded RNA. TBSV affects various plant species, including Nicotianabenthamiana, Arabidopsis thaliana, and other crops. The virus enters plant cells, activating the replication mechanisms of its RNA, which can disrupt the normal functioning of cells and tissues. Infection with the virus causes the destruction of cellular structures, inhibition of metabolic activity and disturbances in the process of photosynthesis. At the same time, changes in the structure and function of the plant's DNA may occur, which in turn can lead to genetic instability and deterioration of resistance to additional stresses [8]. Concurrently, viral infection (e.g., TBSV) intensifies the burden on cellular replication and defense, often elevating reactive oxygen species (ROS) and triggering programmed cell death pathways, thereby compounding genome instability under heat-virus co-stress [5, 9].

Temperature stress has a profound effect on DNA molecules, disrupting their stability and integrity. High temperatures, being an abiotic stress, can cause DNA denaturation, which is the breakdown of hydrogen bonds between a base and a complementary base in a double-stranded DNA molecule. At temperatures above 40–42 °C, the double helix collapses, leading to the formation of single-stranded fragments, which, in turn, can create "hot spots" for subsequent damage [6, 10, 11]. DNA denaturation activates cellular signaling pathways, including single-strand break repair systems and repair of damaged areas. Temperature stress can

also alter the tertiary structure of chromatin, which initiates adaptive mechanisms of cells aimed at maintaining genome stability [12].

Disruption of replication is another important aspect of exposure to temperature stress. At high temperatures, DNA denaturation occurs, preventing the normal functioning of replicative enzymes such as DNA polymerases and disrupting the replication process. This can lead to the arrest of replication and the formation of double-stranded breaks (DSBs), which require intensive repair. Double-stranded breaks are among the most dangerous DNA damages, as they can lead to serious losses of genetic information and disruption of cellular functions [13]. Excessive repair of breaks and the lack of normal replication can also disrupt the cell cycle, especially in critical areas, which causes delays in the passage of the cell cycle and can lead to the accumulation of mutations [14].

Under conditions of extreme stress, the repair mechanisms may be insufficient. The main repair systems, such as excision of damaged bases and repair of double-stranded breaks through restrictases and kinases, are activated, but their effectiveness decreases under severe temperature stress [13]. This process can lead to the accumulation of structural changes in chromosomes, which disrupts the stability of the genome and increases the likelihood of mutagenesis and cell death.

Particular attention should be paid to the combined effects of viral infection and temperature stress, as this interaction can significantly enhance molecular damage. For example, the TBSV virus (Tomato bushy stunt virus) disrupts the balance between viral RNA replication and plant genome replication. Under conditions of viral infection and temperature stress, the formation of DNA breaks increases, which is aggravated by a deficiency of repair mechanisms, leading to increased genomic instability. Viral replication requires significant energy expenditure, which can lead to an increase in oxidative stress, and together with increased temperature, this creates a critical situation for repairing DNA damage.

Analysis of molecular mechanisms

From the above analysis, it can be seen that the effects of temperature stress on plants lead to multiple molecular damages, including DNA denaturation, oxidative damage, and replication disorders. These injuries activate cellular repair mechanisms, but their effectiveness strongly depends on the degree of stress. In turn, viral infections such as TBSV enhance this effect by increasing the load on cellular DNA replication and disrupting the balance between viral and cellular RNA replication.

DNA damage includes single- and double-stranded breaks, base modifications, apurine/apyrimidine sites, and inter-stranded crosslinking [15]. Reactive oxygen species (ROS) react to the effects of viruses and high temperatures, which contribute to oxidative damage to DNA [16, 17]. For example, a significant increase in 8-oxoguanine, one of the main markers of oxidative damage, is observed during heat stress [18].

The combined effects of thermal and viral stress lead to a synergistic effect: increased ROS activity in heat conditions weakens the plant's antioxidant system, while the virus disrupts the regulation of the cell cycle and repair processes [19, 20].

This highlights the importance of research aimed at elucidating deeper molecular mechanisms of interaction between viruses and stressors, as well as developing methods to protect plants from these stresses.

Methods of DNA damage investigation

Modern methods of studying the effects of stress on plants, changes in reactive oxygen species (ROS), and DNA repair processes include several key approaches. One of the most common methods is fluorescence microscopy, which makes it possible to visualize the localization and level of ROS in plant cells using fluorescent sensors. This method allows us to track the dynamics of ROS formation in response to stress, which is important for understanding the mechanisms of cellular adaptation to adverse conditions.

Enzymatic assays also play a significant role in assessing the antioxidant activity of plants. Measuring the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and peroxidase allows us to study the plant's ability to detoxify ROS and its protective mechanisms. These enzymes play an important role in repairing damage caused by oxidative stress, and their activity serves as an indicator of cellular resistance to stress.

Molecular biological methods such as polymerase chain reaction (PCR) and sequencing are widely used to analyze the expression of genes related to DNA repair. Genetic studies reveal changes in the expression of key genes encoding enzymes involved in repairing DNA damage. This makes it possible not only to investigate the mechanisms of repair, but also to identify molecular markers of plant resistance to various stress factors.

In practice, three single-cell/genome-architecture methods are widely used and complementary: the COMET (single-cell gel electrophoresis) for detecting strand breaks and selected base lesions; the TUNEL for apoptosis-associated DNA fragmentation; and the FISH for chromosomal aberrations and spatial genome organization [5, 21, 22, 23].

The COMET method (or Single Cell Gel Electrophoresis, SCGE) is a highly sensitive and effective method for assessing DNA damage at the level of individual cells.

It is based on the electrophoretic migration of DNA fragments forming a characteristic "comet" [21, 24]. In the case of DNA damage, such as breaks, the DNA molecule becomes less compact, which leads to its migration and the formation of a comet-like shape, which is why this method got its name. Cells exposed to stress (for example, radiation, chemicals, temperature stress) are first suspended in an agarose gel. The gel undergoes electrophoresis, while the damaged DNA migrates towards the anode, forming a "tail" (destroyed DNA fragments). After DNA staining using fluorescent dyes, the resulting "comets" are examined under a microscope. The method is used to assess DNA damage caused by various abiotic and biotic stresses, to study mutagenic effects on cells, and to analyze the effectiveness of DNA repair. For plant systems, community guidelines emphasize pre-analytical standardization (tissue type, embedding, lysis, electrophoresis conditions) to ensure reproducibility [25], while recent reviews consolidate plant-specific applications across abiotic and biotic stresses [5, 26]. Beyond laboratory models, the COMET is being used in biomonitoring and sustainability contexts, including climate-change biology and environmental genotoxicity screening [27, 28, 29].

The COMET (Single Cell Gel Electrophoresis, SCGE) method is a highly sensitive tool for assessing DNA damage at the level of individual cells. One of the main advantages of this method is its ability to detect even minor DNA damage, such as single-stranded and double-stranded breaks, as well as base modifications. This method is used to study the effects of various stress factors, such as radiation, chemicals, and temperature stresses [30]. The advantages of the COMET method are its high sensitivity, as it is able to detect DNA damage at the level of individual cells. It is also a simple and affordable method that does not require complex equipment and can be used in laboratories with basic equipment. The COMET method is universal and can be used to assess DNA damage in both plant and animal cells. However, the method has limitations. The method is highly sensitive and applicable for the quantitative assessment of damage [31], but it does not allow distinguishing the types of damage and requires standardization of the conditions [32]. It may also be less informative for assessing the structure of the genome and the detailed localization of genetic damage.

The TUNEL method is used to identify cells in which apoptosis (programmed cell death) occurs. It allows the detection of single-stranded breaks in DNA, which are characteristic signs of apoptosis. During apoptosis, DNA fragmentation occurs in the cell, and free 3'-hydroxyl groups are formed at the ends of these fragments. This method is based on labeling the free 3'-OH ends of DNA breaks [9, 33, 34]. Fluorescent or radioactive dNTP molecules are added to label these ends using the enzyme terminal deoxynucleotidyl transferase (TdT). After that, the marks can be visualized using fluorescence microscopy or other methods. This method is used to analyze apoptosis in cells and tissues, assess DNA damage caused by external factors such as radiation or viral infections, as well as to study the mechanisms of cell death in various organisms [35]. Manipulating pro-/anti-apoptotic regulators (e.g., BAG-family genes) further illustrates how cell-death pathways intersect with stress resilience in crops [36].

The advantages of this method are its ability to accurately detect DNA fragmentation, which is characteristic of apoptosis, and to provide quantitative data on cells undergoing apoptosis [35]. The TUNEL method has a high specificity for apoptosis, which makes it possible to accurately detect DNA damage caused by cell death, and is a powerful tool for studying the mechanisms of apoptosis and progressive DNA damage in cells, which is especially useful when studying the body's response to various stresses. However, this method has limitations. It does not allow detecting DNA damage in living cells, as it requires sample fixation. In addition, it only tests for the same type of DNA damage (breaks at the ends of DNA fragments), which limits its use for analyzing other types of damage. TUNEL is sensitive to the late stages of apoptosis, but can give false positive results in the presence of necrotic lesions [37].

The FISH (fluorescent in situ hybridization) method is used to visualize specific regions of chromosomes.

The method is particularly useful for detecting chromosomal aberrations and genome instability [22, 23, 38, 39]. This method is based on the specific binding of fluorescently labeled probes to the corresponding DNA or RNA regions. DNA is fixed in cells or tissues, which is then denatured to separate the

 ISSN 3080-6836 (Print) ISSN 3080-6844 (Online)

chains. Specific single-stranded oligonucleotides labeled with fluorescent dyes are added to the sample and bind to complementary DNA regions. Measuring fluorescence using a microscope makes it possible to localize these areas and examine their distribution throughout the cell. The FISH method is used to study the structure of chromosomes, including locating genes and other sequences, as well as to evaluate chromosomal abnormalities such as deletions, duplications, and translocations. In addition, the method is used to study the distribution of specific genes or viral genomes in cells [40]. Methodological notes for cereals and other taxa highlight practical considerations for denaturing vs non-denaturing protocols and sample quality control [41].

The FISH method (fluorescent in situ hybridization) allows us to study the spatial organization of the genome, as well as analyze the localization of specific genes and viral genomes. It has high accuracy, as it allows localization of certain DNA sequences directly in cells or tissues. This method is widely used to detect chromosomal abnormalities such as deletions, duplications, and translocations [40]. The FISH method allows precise localization of specific DNA regions in chromosomes, which provides a deep understanding of the structure of the genome. It can be used in both plant and animal cells, providing information about chromosomal abnormalities such as deletions, duplications, and translocations, which is important for genetic research and diagnosis. However, the method has limitations. It requires high precision in sample preparation [42] and can be difficult to work with degraded samples. In addition, the method can be time-consuming and require highly qualified personnel to interpret the results. FISH is also limited in its use to detect DNA damage if it is not located in the area of the genes or chromosome regions of interest.

Integrated use under combined stress conditions. COMET, TUNEL, and FISH together provide a multi-angle assessment of stress impacts: COMET detects overall strand-break burden and oxidative lesions; TUNEL quantifies apoptosis-associated DNA fragmentation; FISH identifies structural chromosomal rearrangements and spatial genome alterations. In combined heat-virus scenarios in model hosts such as N. benthamiana, such an integrated panel can reveal elevated DNA migration (COMET), extensive TUNEL positivity in affected tissues, and FISH-detectable rDNA/centromere instability—linking molecular lesions to cytological outcomes [5, 7, 23].

These methods have different applications in molecular biology and genetics, and each provides unique information about DNA damage and genome structure. The COMET method is suitable for assessing DNA damage at the cellular level, TUNEL helps to study apoptosis and DNA fragmentation, and FISH helps to analyze the localization of genetic sequences and chromosomal abnormalities [43, 44]. In the context of combined stress, the use of a combination of all three methods is the most informative [45]. For example, when analyzing Nicotiana benthamiana exposed to thermal and viral stress, COMET revealed a sharp increase in DNA migration, which indicates the presence of multiple breaks [45], whereas TUNEL confirmed active apoptosis in mesophyll cells [46], and FISH showed rDNA instability and signals of loss of centromeric regions [47].

However, each method has its limitations, such as the need to prepare high-quality samples or the difficulty in interpreting the results, which requires knowledge and experience from the researcher.

Conclusion

Combined abiotic and biotic stresses, including heat and viral infection, impose multi-layered burdens on plant genomes, from oxidative base damage and strand breaks to large-scale chromosomal instability. Applying COMET, TUNEL, and FISH in concert yields complementary evidence spanning single-cell DNA damage, apoptosis-associated fragmentation, and chromosomal architecture, informing mechanism-driven strategies for improving stress resilience [5, 6, 34, 48].

The study showed that the combined effects of various stress factors, such as temperature stress and viral infections, significantly affect plants at the molecular level, which puts cells in a critical position where defense mechanisms such as heat shock proteins and DNA repair systems may not be able to cope with damage. This highlights the importance of research aimed at elucidating deeper molecular mechanisms of interaction between viruses and stressors, as well as developing methods to protect plants from these stresses.

Using the COMET, TUNEL, and FISH methods allows you to obtain comprehensive information about the types and extent of damage. The COMET method effectively evaluates DNA damage at the level of individual cells, allowing us to analyze the effects of various stressors, including radiation, chemicals, and temperatures. The TUNEL method makes it possible to assess apoptosis-related damage and analyze the mechanisms of cell death, while the FISH method allows for detailed investigation of the genome structure, identification of chromosomal abnormalities and gene localization. Each of these methods has its advantages and

disadvantages, and the choice of a specific approach depends on the objectives of the study, the type of samples and available resources.

Thus, there is a comprehensive approach to stress research, including viral infections and temperature stress, and these data are critically important for understanding plant resistance mechanisms and developing stress-resistant varieties [2, 15, 49, 50].

Acknowledgments

This work was carried out within the framework of the grant project AP19679597 "Study of multisystem plant defense under combined stress conditions".

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Z. Turarbekova*** (Corresponding author) – Conceptualization; Methodology (overall comparative framework; COMET protocol standardization); Investigation (sample preparation and COMET runs); Visualization (figures and workflow diagram); Writing – original draft; Writing – review & editing; **N. Iksat** – Methodology (TUNEL assay setup and optimization); Investigation (TUNEL staining and microscopy); Validation (assay controls); Writing — review & editing; **A. Madirov** – Methodology (FISH probe design and hybridization workflow); Investigation (FISH imaging); Formal analysis (image quantification); Data curation; Writing — review & editing; **A. Tuyakbayeva** – Methodology (plant growth conditions and combined stress treatments — heat and viral inoculation); Investigation (stress application and sampling); Validation (biological replicates and controls); Data curation (metadata and sample tracking); Writing — review & editing; **Z. Masalimov** – Supervision; Conceptualization (study design and scope); Project administration; Funding acquisition; Resources (laboratory infrastructure and reagents); Writing — review & editing.

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Абиотикалық және биотикалық кернеулер кезінде өсімдіктердегі ДНҚ-ның зақымдануын бағалау үшін COMET, FISH және TUNEL әдістерін салыстырмалы талдау

Өсімдіктердің ДНҚ-сын әртүрлі стресс факторларының әсерінен зақымдануын зерттеуге бағытталған зерттеулер қазіргі заманғы молекулалық биология мен генетиканың маңызды бағыттарының бірі. Соңғы онжылдықтарда өсімдіктердің абиотикалық және биотикалық стрестерге молекулалық реакцияларын егжей-тегжейлі талдауға мүмкіндік беретін әдістер белсенді дамып, өсімдіктердің қолайсыз жағдайларға бейімделу механизмдерін тереңірек түсінуге жол ашты. Мұндай зерттеулердің негізгі аспектілерінің бірі — генетикалық материалдың зақымдану деңгейін бағалау, себебі бұл өсімдік жасушалары мен тіндерінің қалыпты қызметін бұзуға айтарлықтай әсер етеді. Әсіресе, жоғары температура мен вирустық инфекциялар (мысалы, Tobacco bushy stunt virus — TBSV) сияқты стресс факторларының біріктірілген әсеріне ерекше назар аударылады, себебі олар ДНҚ-ның тұтастығын бұзып, жасушалық процестердің қалыпты жүруіне кедергі келтіруі мүмкін. Бұл өз кезегінде ДНҚ-ны қалпына келтіру, негізгі гендердің белсенділігі мен өсімдіктердің физиологиялық және морфологиялық ерекшеліктеріне әсер етуі мүмкін. Мақалада біріктірілген стресс жағдайларында өсімдік ДНҚ-сындағы зақымдануды бағалауда жиі қолданылатын үш әдіс қарастырылды: COMET (сілтілі гельдік электрофорез), TUNEL (терминальді дезоксинуклеотидилтрансфераза арқылы нуклеотидтердің тізбекті жалғануы) және FISH (флуоресценттік in situ гибридизация). Бұл әдістер ДНҚ зақымдануын кешенді түрде талдауға, сондай-ақ вирустық және температуралық стресс

жағдайларына ұшыраған өсімдіктердегі физиологиялық және жасушалық өзгерістермен байланысын зерттеуге мүмкіндік береді. Бұл зерттеудің мақсаты — өсімдіктерге абиотикалық және биотикалық стресс факторларының әсері кезінде ДНҚ-ның зақымдану деңгейін бағалау үшін COMET, FISH және TUNEL талдау әдістерін қолдану мүмкіндіктерін зерттеу. Зерттеу бұл әдістердің тиімділігін талдауға, сондай-ақ өсімдік нысандарымен жұмыс істеу кезінде олардың артықшылықтары мен шектеулерін анықтауға бағытталған.

Кілт сөздер: TBSV, Nicotiana benthamiana, біріктірілген стресс, ДНҚ зақымдануы, тотығу стресі, ДНҚ репарациясы, COMET-талдау, TUNEL-талдау, FISH-гибридизация.

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Сравнительный анализ методов COMET, FISH и TUNEL для оценки повреждений ДНК растений при абиотических и биотических стрессах

Исследования, направленные на изучение повреждений ДНК у растений под воздействием различных стрессовых факторов, являются важным направлением современной молекулярной биологии и генетики. В последние десятилетия наблюдается активное развитие методов, позволяющих детально анализировать молекулярные реакции растений на абиотические и биотические стрессы, что значительно углубляет понимание механизмов их адаптации к неблагоприятным условиям. Одним из ключевых аспектов таких исследований является оценка повреждений генетического материала, поскольку они играют важную роль в нарушении нормального функционирования клеток и тканей растений. Особое внимание уделяется комбинированному воздействию стрессовых факторов, таких как высокая температура и вирусные инфекции, например заражение вирусом кустистой карликовости томата (*Tobacco bushy stunt virus*, TBSV), способных существенно нарушать целостность ДНК и нормальные клеточные процессы. Это, в свою очередь, может приводить к изменениям в активности ключевых генов, нарушению процессов репарации ДНК, а также оказывать влияние на физиологические и морфологические характеристики растений. В данной статье рассмотрены три метода, активно применяемые для оценки повреждений ДНК в условиях комбинированного стресса: COMET (щелочной гель-электрофорез), TUNEL (метод терминальной дезоксинуклеотидилтрансферазы) и FISH (флуоресцентная *in situ* гибридизация). Эти методы позволяют проводить комплексный анализ повреждений ДНК, а также исследовать их взаимосвязь с физиологическими и клеточными изменениями у растений, подвергшихся воздействию вирусного и температурного стресса. Целью данного исследования является изучение перспектив применения методов COMET, FISH и TUNEL для оценки степени повреждения ДНК растений под влиянием абиотических и биотических стрессов.

Ключевые слова: TBSV, Nicotianabenthamiana, комбинированный стресс, повреждение ДНК, окислительный стресс, репарация ДНК, COMET-анализ, TUNEL-анализ, FISH-гибридизация.

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