Radiation monitoring with plant-based biotas and an automated micronucleus scoring approach

F. d'Errico[1,*], F. Barco[1], T. Butini[1], M.G. Cascone[1], R. Ciolini[1], M. Quattrocchi[3], E. Rosellini[1],
T. Hulber[4], J.A. Torres Novaes[2], M.N. Xavier[2], S. de Souza Lalic[2]
[1]School of Engineering, University of Pisa, Italy
[2]Department of Physics, Federal University of Sergipe, Brazil
[3]Department of Radiology, St. Luca Hospital, Lucca, Italy
[4]Radosys, Budapest, Hungary

(*) francesco.derrico@unipi.it

Abstract— **In this work, we investigated the response of the stem cells from the roots of Allium Cepa (meristems) to ionizing and non-ionizing radiations of different qualities. Allium cepa (Onion) is a well-established in-vivo standard model, widely used in cytogenetic studies for different environmental pollutants. Endpoints, such as chromosomal aberrations (CAs), micronuclei (MNs), and disturbance in the mitotic cycle of root meristematic cells of onion are frequently used to determine the cytotoxic and genotoxic potential of different environmental pollutants. Traditionally, these studies have been carried out to assess chemical toxicity, while the toxicity of ionizing radiation has been studied less extensively. We also examined the repurposing of a previously described optical microscope system originally designed for automated non-fluorescent micronucleus (MN) scoring in binucleated peripheral lymphocytes. The microscope system relies on hardware and software layers in parallel in order to optimize the performance in automated MN scoring.**

Keywords —**Biota, micronucleus, ionizing radiation, non-ionizing radiation.**

I. INTRODUCTION

THE analysis of cytotoxic effects of ionizing radiation in the flora and fauna is of interest in agriculture, ecology, health care, and space exploration. Humans are continuously exposed to radiation from a variety of sources throughout their lives, such as solar radiation, natural background radiation, or medical devices. Typically, absorbed dose assessment is done using physical dosimeters, but these tools are not commonly available. This has led to the need to develop new strategies to assess the damage, especially to DNA, caused by such radiation. There was a need to identify parameters (endpoints) that would allow the effects of radiation on cells, particularly on genetic material, to be assessed and to correlate these effects with the dose received. The relationship between endpoints and absorbed dose is known as biodosimetry [1], and relies on the quantification of biomarkers such as micronuclei and chromosomal aberrations to assess the dose received by a subject.

The effects of ionizing radiation in plants [2] may provide an efficient warning system to avoid harm to human health. Plants are unlikely to exhibit the same stochastic effects of ionizing radiation that are observed in animals. However, higher plants present effects that may mimic those of other biological systems, since the critical target is the DNA, common to all organisms. Indeed, previous studies have shown that chemical genotoxic agents produce comparable effects in plant and animal systems in terms of genetic abnormalities [3]. Additionally, these studies on so-called plant-based biotas avoid the controversial use of animal models. Thus, they represent a new frontier of radioecology, an emerging field of environmental ecology that investigates the presence and the effects of radioactivity in Earth's ecosystems. The investigated effects are typically DNA damage visualized as chromosomal aberrations when cells divide, or as the presence of micronuclei in inter-phasic cells.

The present study focuses on biodosimetry of ultraviolet (UVB and UVC) and ionizing radiation (high and low linear energy transfer, LET) particularly alpha particles and X-rays, using a plant organism, Allium cepa (commonly known as "onion"), as an alternative method to animal models. Among biological species, Allium cepa is a good bioindicator for damage estimation because of its high sensitivity not only to chemicals but also to radiation [4-7]. The use of this organism is very important because it is a diploid species ($2n = 16$) with a low number of large chromosomes, which makes it easy to observe defects occurring in these chromosomes. In addition, it has a predominantly aqueous chemical composition, similar to that of human soft tissue.

The genotoxicity of radiation was studied using the micronucleus test [5]. The micronucleus test consists of microscopic analysis of cells during mitosis, taken from an organism exposed to a potentially mutagenic agent. Its purpose is to identify the presence of scattered fragments of DNA in the cytoplasm that are not incorporated into the nucleus during the final stages of cell division due to the mutagenic agent.

The experimental work based on the use of Allium cepa seeds started from a known method [7] but was optimized and standardized through the utilization of a device that mechanically compresses the apical meristem of Allium cepa. This technique allows obtaining the required monolayer of cells for observing DNA damage (micronuclei, aberrations, etc.), thereby enhancing the reproducibility of the entire procedure.

II. MATERIALS AND METHODS

The method for preparing the samples necessary to perform damage analysis resulting from irradiation (particularly for the identification of micronuclei and aberrations) can be divided into five main phases: germination of Allium Cepa seeds, irradiation, slide preparation, sample squashing, and microscope analysis. All operations are carried out under sterile conditions, operating within a laminar flow hood.

After sterilization, the sample preparation procedure begins as follows:

Germination: In this study, organic onion seeds of the Tropea variety were used. The seeds are placed, maintaining a distance of 1 cm from each other, inside Petri dishes in which moistened filter paper sheets are placed as a culture medium. Finally, the plates are sealed and placed in an incubator at 25 ±1 °C for 3 days to obtain sprouts approximately 3-4 mm in length.

Irradiation: For irradiating the meristems of Allium Cepa, a lamp consisting of three UV tubes, one for each wavelength (254 nm, 302 nm, 365 nm), was used. Dosimetric studies were conducted using three radiometers. In the first phase, the three detectors were placed at the same distance from the lamp to obtain the normalization point of the radiometers' response. Subsequently, the irradiance profile was evaluated, observing that the measurements follow a 1/r profile, consistent with the behavior of a linear source. For UVC irradiation, the plates were positioned at a distance of 7.3 cm from the lamp, corresponding to an irradiance of 0.818 mW/cm², and doses of 123 mJ/cm², 245 mJ/cm², 368 mJ/cm², and 491 mJ/cm² were administered. For UVB irradiation, the plates were positioned at a distance of 13 cm, resulting in an irradiance of 0.690 mW/cm², and doses of 104 mJ/cm², 207 mJ/cm², 311 mJ/cm², and 414 mJ/cm² were administered.

Alpha irradiation was conducted at the laboratories of the University of Pisa using a 241Am source with a dose rate of 7.92 mGy/min. The absorbed doses were 20, 40, 60, and 80 mGy. Five samples were analyzed for each dose point.

Regarding X-rays, the experimentation was conducted at the San Luca Hospital Center in Lucca, where a linear accelerator was used to produce 6 MV X-ray photons with a dose rate of 100 monitor units (MU)/min. MU is defined as the monitoring unit, representing the time required for the accelerator's monitor chamber to deliver a certain dose to the tissue. The system is calibrated so that 100 MU corresponds to 1 Gy. The absorbed doses were 150, 300, 450, and 600 mGy, and these values were obtained based on the assessment of relative biological effectiveness. Literature data indicate that, at the same dose, there is an approximately one-order-of-magnitude difference in the effect between high and low linear energy transfer (LET) radiation. Therefore, doses for X-rays were chosen to be approximately one order of magnitude higher than those for alpha particles.

After irradiation, the germinated seeds are returned to the incubator for 24 hours. This time is necessary to allow the cells in the apical portions of the sprouts to complete at least one cell cycle and permit the potential formation of micronuclei and chromosomal aberrations. After 24 hours, the sprouts are placed in Falcon tubes containing the fixative Carnoy (which halts the cell cycle) and left for 24 hours at a controlled temperature. The final phase involves transferring the seeds to Falcon tubes containing 70% ethanol, where they are stored at a temperature of 4 ±1 °C until they are used for slide preparation.

Slide preparation: The germinated seeds are placed inside a Falcon tube with 1M HCl and placed in a water bath at a temperature of 60°C for 5 minutes. Subsequently, the seeds are covered with distilled water for 10 minutes to hydrate them, and then soaked in drops of acetic acid for 3 minutes to facilitate the breaking of the cell wall [8]. Finally, the sprouts are stained using acetic orcein for 15 minutes.

Squashing of the apical meristem of Allium Cepa: Each seed is placed on a glass slide, and using a scalpel, the mitotically active apical region is separated from the rest of the sprout. It is then covered with a coverslip. The squashing of the meristem, traditionally done manually by pressing the slide with the thumb, was instead performed using a device designed and developed for this thesis work to standardize the procedure and obtain perfectly reproducible samples. After squashing, the slides are sealed and stored in a refrigerator at a temperature of 4 ±1 °C.

Reading of the slides: The slides, removed from the refrigerator 20-30 minutes before reading, are placed under a Nikon Eclipse TS100 optical microscope and analyzed at a total magnification of 400x. The analyzed sample is considered valid for analysis if it contains 1000 cells [6], as the micronucleus test aims to determine the number of cells with micronuclei out of 1000 cells counted. To reduce time and minimize the risk of errors during reading, a double reading approach was implemented, one on the computer where the counting of 1000 cells is performed using a photo of the region of interest, and one under the microscope, necessary to discriminate micronuclei and chromosomal aberrations.

A device for crushing sprouts was created with the aim of "engineering" and standardizing one of the most delicate phases of the procedure, which allows obtaining a monolayer of cells to be subsequently analyzed under a microscope. The traditional method involves compressing the sprouts by crushing them with the thumb, without any control over the applied pressure (which can vary from time to time).

The functioning of the device is as follows: the glass slide with the sprout is placed in a dedicated housing on a cart. This cart, guided by two linear guides, moves under a bridge to which the system for applying force is hinged. The force application system consists of a roller connected to an adjustable spring with a nut. By compressing or loosening the spring, the force that the roller will apply to the glass slide during the cart's passage can be modulated.

To evaluate the actual force applied by the roller, a scale has been designed and implemented within the structure. It consists of a load cell mounted near the cart, which contains the electronic circuitry inside. This load cell is controlled by a dedicated chip (HX711), which is connected to the "brain" of the system, namely a compatible Arduino Nano board. The board is also connected to a display for visualizing the result and a button for taring.

Besides the manual microscope mentioned above, an automated system was also examined relying on hardware and software operating in feedback-mode in order to optimize the performance in automated MN scoring [9]. A MN assay slide is first scanned, then cells and micronuclei are automatically identified. The image processing module of the microscope is an integral part of the system and works in synergy with the image acquisition. This module identifies the contour of the objects of interest (namely cell, nucleus and MN) stepping through the scanned fields of view of a slide. This module also provides feedback to the illumination and focusing functions. The image segmentation process of a single field of view can be divided into three subsequent steps. Cells are identified first, then nuclei and finally micronuclei. Each step of the segmentation comprises two substeps: first, the algorithm creates a pool of candidate objects that still includes a significant number of false positives. Afterward, in the so-called classification step, the artifacts are eliminated based on their geometrical, morphological and staining intensity features. The device was previously demonstrated to offer the accuracy of visual scoring aided by minimal user interaction. The possibility of fully automatic scoring with an accuracy suitable for triage purposes is also available in case a surge of cases is to be managed.

III. RESULTS

A. Alpha particle and X-Ray Irradiations

The parameters considered for evaluating the damage resulting from irradiation were the number of cells with at least one micronucleus and the number of cells with multiple micronuclei. Analyzing the results obtained on the apical meristems of Allium cepa (Figure 1), it can be observed that both in the case of alpha particles and X-rays, the number of cells with micronuclei increases with the absorbed dose. Therefore, a dependence of the number of cells with micronuclei on the absorbed radiation dose can be hypothesized.

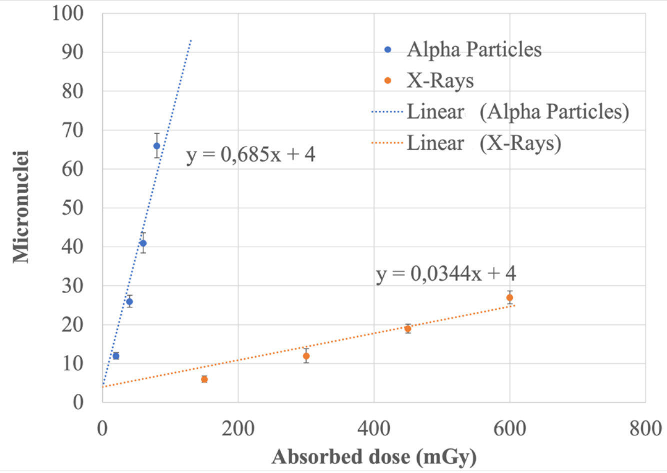

Fig. 1. Number of cells with micronuclei after alpha particle and X-Ray irradiations.

In particular, it is observed that in the case of alpha radiation, there is a much more rapid growth of the number of cells with micronuclei, concentrated in a narrower range of absorbed doses, while in the case of X-rays, the growth is much slower and occurs over a wider range of doses. This result is in perfect agreement with the fact that high linear energy transfer (LET) radiation (alpha particles), as known, is capable of causing more DNA damage compared to low LET radiation (X-rays).

This confirmation is evident from the analysis of the ratio between the two plotted lines (which do not follow the actual data but are drawn as a guide for visualization). Considering the ratio between the slopes, it is possible to define a ratio of approximately 20.

In the determination of the effective dose of radiation, the "radiation weighting factor" is introduced, assigned based on their potential harm. Low LET radiation is associated with a value of 1, while high LET radiation is assigned a weighting factor of 20. Therefore, the ratio between the factors related to high and low LET radiation is precisely 20, as observed in the ratio between the slopes of the previously described lines. This confirms the reliability of the obtained results.

Additionally, as a parameter, the number of cells with two or more micronuclei was analyzed. The presence of a higher number of DNA defects within a cell can be a symptom of greater damage to the genetic material. Once again, it can be observed that alpha radiation is capable of generating a higher number of "polymicronucleated" cells compared to X-rays. Specifically, considering all the analyzed samples, 40 cells with a double micronucleus (including one with three micronuclei) were counted out of a total of 20,000 cells counted in the case of alpha particles, while only two cells with a double micronucleus were counted out of 20,000 cells in the case of X-rays.

B. Ultraviolet B and C Irradiations

To compare the two types of UV radiation used, Figure 2 shows the variation in the number of cells with micronuclei as a function of absorbed dose. The two lines in the figure do not represent the actual interpolation of the data but are shown for visualization purposes. Analyzing the trends of these lines, it can be observed that both UVC and UVB irradiation lead to an increase in the number of cells with micronuclei as the dose increases, which is considered an indicator of radiation-induced damage. The two trends are comparable, indicating that there does not seem to be a microscopic difference between the two types of radiation.

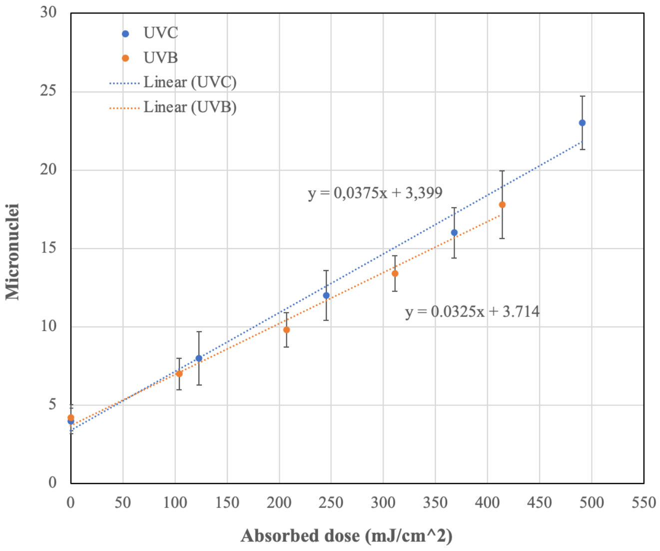

Fig. 2. Number of cells with micronuclei after UV irradiation.

It can be presumed, therefore, that the difference in damage caused by UVB and UVC radiation is not related to the deposited dose but rather to the type of action exerted by these radiations on the cells. What has been observed is a difference in terms of macroscopic damage: for UVC radiation, cells irradiated to 491 mJ/cm^2 show intact cytoplasmic membrane integrity (Fig. 3), this holds even at higher doses and the only detected damage is at the microscopic level involving DNA.

On the other hand, for UVB radiation, cells irradiated to a similar dose of 414 mJ/cm^2 show altered morphology characterized by cytoplasmic rupture and nucleus degeneration (Fig. 4).

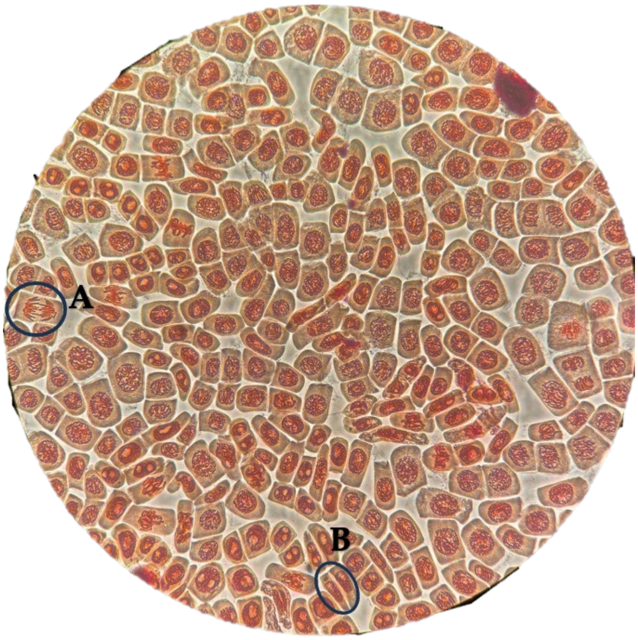

Fig. 3. Allium cepa meristems irradiated to 491 mJ/cm^{2}of UVC radiation, a bridge (A) and a micronucles (B) can be observed in the image.

Fig. 4. Allium cepa meristems irradiated to 491 mJ/cm^{2}of UVC radiation, altered morphology (C) and nucleus degeneration (D) can be observed in the image.

By observing the cell morphology after irradiation, it can be inferred that UVC radiation primarily exerts a particle-like effect, characterized by targeted ionizing action on DNA and photochemical action. In contrast, UVB radiation predominantly exhibits a photochemical action, characteristic of this type of radiation, which leads to morphological alterations of the entire cell.

Indeed, UV radiation can damage DNA in two ways: directly and indirectly [10]. Direct DNA damage primarily involves the creation of dimerized pyrimidines, and this type of damage is likely the most significant factor in causing cancer. This dimer formation process depends on the absorption of UV light by the DNA bases, particularly in the UVB and UVC ranges causing the formation of electronic excited states. For instance, germicidal lamps emitting light at 254 nm effectively cause the dimerization of pyrimidines. However, this process is still quite effective in the UVB range (280–320 nm). The dimerization process leads to the formation of cis-syn cyclobutane pyrimidine dimers, which are the most common abnormalities in double-stranded DNA, or pyrimidine (6–4) pyrimidone photoproducts, which are the second most frequent DNA lesions in the UVB range. In addition to the formation of pyrimidine dimers, UVA and UVB indirectly contribute to the generation of oxidized DNA base damage, often triggered by light-absorbing molecules, known as chromophores, leading to photosensitization reactions.

IV. CONCLUSIONS

By comparing the results obtained from alpha radiation in this study with those documented in the literature [7], it can be concluded that Allium cepa has the potential to serve as a viable alternative model for detecting genotoxic effects from low doses of alpha radiation, instead of relying on animal models. Furthermore, this plant model has demonstrated the ability to show distinct effects caused by different types of radiation, indicating its potential to differentiate between various linear energy transfer (LET) values.

Furthermore, Allium cepa shows promise as a substitute for animal models in understanding X-ray radiation-induced biological effects. However, because this study represents a novel exploration not previously conducted by others, further investigation and data collection are needed to validate the results and hypotheses obtained.

The results of this study also indicate, for the first time, that Allium cepa has potential as a model for evaluating the biodosimetry of ultraviolet radiation. It was observed that both UVC and UVB radiation result in an increase in the number of cells with micronuclei as the dose increases. UVC radiation primarily acts on DNA at the microscopic level, whereas UVB radiation predominantly induces photochemical actions that alter cell morphology. These observed effects motivate further research, including analysis of the effects of UVA radiation, to understand the full spectrum of ultraviolet radiation, and opens the possibility of performing analysis of strictly wave radiation, such as electromagnetic radiation.

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