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# COMET ASSAY OPTIMIZATION WITH ALLIUM CEPA AS AN INDICATOR FOR ECOTOXICOLOGICAL USAGE

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Abstract

**The pollutants discharged to the environment cause a real threat for all types of the ecosystem. One of the most important recalcitrants directed to the environment is hospital wastewater, harmful because of both, microbiological and ecotoxicological potential (they consist of a large amount of drugs and its metabolites). These substances can cause cells damage at the genetic level and they can be dangerous to the human and animals life. To estimate ecotoxicological potential of environmental mutagens comet assay is often used. It is cheap, fast, simple and sensitive technique enabling to estimate the damages of genetic material and the effectiveness of its treatment. Fast increase of the hospital wastewater volumes and changes of its composition cause the necessity of fast and reliable testing systems introduction for potentially genotoxic compounds analysis. Such tests should be performed on easy to obtain and inexpensive eucaryotic indicators leading to their common usage for mutagenicity and toxicity test. The eukaryotic organism fulfilling these requirements is an onion – *Allium cepa*. Due to its sensitivity and good correlation with mammalian cells results *A. cepa* is an effective tool for routine environmental mutagens genotoxicity tests and they can be used in comet assay. Although, it is still not commonly used for ecotoxicological research. The comet test with *A. cepa* gives the possibility of fast and not – expensive analysis of hospital wastewater toxicity assessment. The comet assay is relatively easy to perform, but it requires individual protocol optimization for a particular experiment. That was the reason why the aim of this study was to optimize comet assay on the basis of already published protocols using *Allium cepa* meristematic root cells as a good indicator for hospital wastewater ecotoxicological potential estimation.**

Streszczenie

**Ścieki przedostające się do środowiska stwarzają realne zagrożenie dla każdego ekosystemu. Jednym z ważniejszych zanieczyszczeń kierowanych do środowiska są ścieki szpitalne, będące zagrożeniem zarówno mikrobiologicznym, jak i ekotoksykologicznym, ze względu na znaczną zawartość leków i ich metabolitów. Związki te mogą powodować uszkodzenia komórek na poziomie ich materiału genetycznego, a co za tym idzie, stanowić poważne zagrożenie zdrowia i życia ludzi. Do szacowania potencjału ekotoksykologicznego mutagenów środowiskowych stosuje się często test kometowy (ang. *comet assay*). Jest on tanią, szybką, prostą i czułą techniką, pozwalającą ocenić uszkodzenia materiału genetycznego oraz efektywność jego naprawy. Wzrost ilości i szybkie zmiany składu zanieczyszczeń szpitalnych powodują konieczność opracowania szybkich i wiarygodnych systemów testowych do badań wpływu substancji potencjalnie genotoksycznych. Analizy tego typu powinny być wykonywane na łatwo dostępnych i tanich indykatorach eukariotycznych, co ułatwi ich powszechne wprowadzenie i wykorzystanie w testach mutageniczności i toksyczności substancji badanych. Organizmem eukariotycznym spełniającym te wymagania jest cebula zwyczajna (*Allium cepa*), która ze względu na czułość i korelację z wynikami uzyskanymi z użyciem ssaczych komórek, może stanowić alternatywę dla testów na komórkach zwierzęcych. Wciąż jednak nie jest ona powszechnie stosowana w analizach ekotoksykologicznych. Zastosowanie testu kometowego z użyciem komórek *A. cepa* daje możliwość szybkiej i taniej analizy toksyczności ścieków szpitalnych. Pomimo swej prostoty wymaga on jednak indywidualnego dopracowania metodyki do określonego eksperymentu. W związku z powyższym podjęto próbę zoptymalizowania testu kometowego w oparciu o analizę dostępnych w bibliografii procedur badawczych w celu wykazania przydatności komórek merystematycznych korzeni *Allium cepa* jako indykatora toksycznego wpływu ścieków szpitalnych na komórki eukariotyczne.**

Keywords: ***Allium cepa*; Comet assay optimization; Hospital wastewater toxicity; Genotoxicity.**

## 1. INTRODUCTION

Ecosystem degradation caused by anthropogenic pollution can be evaluated with chemical and biological analyses due to the estimation of the changes and to find a proper way of its treatment. The occurrence and fate of pharmaceuticals in wastewater, surface water and ground water is nowadays an important point of environmental discussion [1, 2]. Special attention is now paid to the hospital wastewater, which are a source of antibiotics, iodized X-ray contrast media, disinfectants, heavy metals, AOX (Adsorbable Organic Halogens) and cytostatic agents [3]. Although the hospitals are the main source of such substances in the environment it should be underlined that also ambulatory service as well as households also release large amount of pharmaceuticals. A large set of data indicates that hospital wastewater is characterized by 15 times higher ecotoxicological potential than municipal wastewater so it is reasonable and necessary to perform analysis of their mutagenic, cytotoxic and genotoxic influence on the organisms [1].

In ecotoxicological research it is important to estimated genotoxic potential for both, the mixtures and single chemical compound. The pollutants directed to the environment could be harmful to all sorts of the ecosystems. Before the changes caused by the toxicants will be visible at the level of the organisms, at first they appear at the level of the cell or it structures. The decrease of the environmental pollution requires the development of eukaryotic test systems to study potentially genotoxic influence of the substances to the plant and animal cells. The animal cells, such as mammalian ones seems to be ideal for genotoxicity assessments. But also plant cells appear to be useful in genotoxicity analysis [4], especially these presenting the genotoxicity tests results convergent with animal cells. Environmental monitoring data collection using plants as indicators could be handy for environment risk assessment [5].

An onion (*Allium cepa*) is often used as ecotoxicological indicator. *A. cepa* possesses several features important for a potential genotoxicity indicator, such as: accessibility, cost-effectiveness, ease to grow in relatively short time [6]. Due to the test sensitivity and results well correlating with mammalian cells analysis *A. cepa* is an effective tool for routine genotoxicity tests in the environment [7].

Comet assay, known also as single cell gel electrophoresis (SCGE) is known to be a technique enabling the level of DNA damage estimation and their repairing efficacy at the level of single cells [4]. Comet assay is a sensitive, simple, fast and relatively inexpensive test, often used for genotoxicity of chemicals assessment [6]. By definition, genotoxicity is an ability of chemical substances for DNA damage induction, somatic cells mutation or the other changes which can be passed to the next generation [8].

Comet assay consists of several steps. After the exposition to potentially genotoxic compound, cells nuclei are isolated and submerged in agarose on microscopic slides. For DNA spreading in the area of its damage relaxation procedure is performed. Free DNA segments during the electrophoresis migrate to anode. DNA leaking from the damaged nucleus is presented as the tail of the comet, which length and intensity depends on the level of the damages occurred. DNA staying in the nucleus is non damaged (the head of the comet). The estimation of DNA damage is performed with biocomputing methods [9].

Regular SCGE let to detect only a particular level of DNA damages: single strand break (SSB), double strand breaks (DSB), DNA breakages and the changes appearing in alkaline environment [8]. The sort of the damage detected and the sensitivity of the method depends on the range of pH used (9.5-13.5). In neutral environment with pH = 9.5 it is possible to detect only DSB. More alkaline environment, with pH ranged between 10-13 enables to analyze SSB. The most popular comet test in strongly alkaline environment (pH > 13) is used for SSB and DSB detection and enables to estimate changes instable in alkaline environment. This type of DNA damage in pH > 13 is converted to SSB and presented in this way. Strongly alkaline SCGE gives also more intense comet pictures [9].

Although SCGE is widely used for eukaryotic cells it requires individual adjustment for each experiment. The laboratory protocol used depends on the mutagen and test system chosen for a particular experiment. Except the solutions used influence also the lab equipment is relevant for the test performance [10]. Slightly different relaxation time, or the other parameters can be an obstacle for nuclei isolation, the picture of the comet or proper results obtainment. So the optimization of the method for the particular test is required.

Because the comet assay is the procedure in which the result can be estimated only after the slides visualization the preparation of the optimal protocol was difficult and time consuming. The research performed in the beginning with all procedures analyzed separately showed no positive results in comets and

nuclei obtainment in genotoxicity assessment test of real hospital wastewater using *A. cepa* root meristem cells as an indicator. At the most slides only the artifacts instead of the nuclei were observed. If the nuclei were obtained their picture was blurred, probably because of the failure of the nuclei isolation. It is possible that during the washing procedure most of the isolated nuclei were washed away. Also the electrophoresis buffer and the separation procedure could cause the problems during the procedure, because in the beginning we used buffer not freshly prepared and the separation was performed at the room temperature, not on ice.

Because of the previous failure of the comet assay protocol for this material the aim of this experiment was to optimize SCGE protocol for particular type of indicator (*A. cepa*) and potentially genotoxic compound (hospital wastewater) on the basis of four protocols from bibliography which failed in the previous experiment with the same material. Only after comparing and correcting the particular steps of the procedure it was possible to obtained a positive result of the test.

## 2. MATERIALS AND METHODS

### 2.1. Biological material preparation

For this experiment commercially used healthy onions, similar in size, were used. Before the experiment they were kept in a dry and dark place. Onions were grown hydroponically in static condition. The experiments were performed with wastewater samples from St Joseph's Hospital for Phthisiatry and Lungs Diseases located in Pilchowice (Upper Silesia, the south of Poland). Positive control was 100% hospital wastewater, negative control – distilled water.

### 2.2. Comet assay method

During the optimization for protocols of SCGE were compared: [4], [6], [11] and [12], in neither of which satisfactory results for our experiment were obtained. All the protocols, except [4], performed analysis on *A. cepa*. The modified protocol was created on the basis of for protocols mentioned above, performed in parallel, analyzed and compared in order to find the

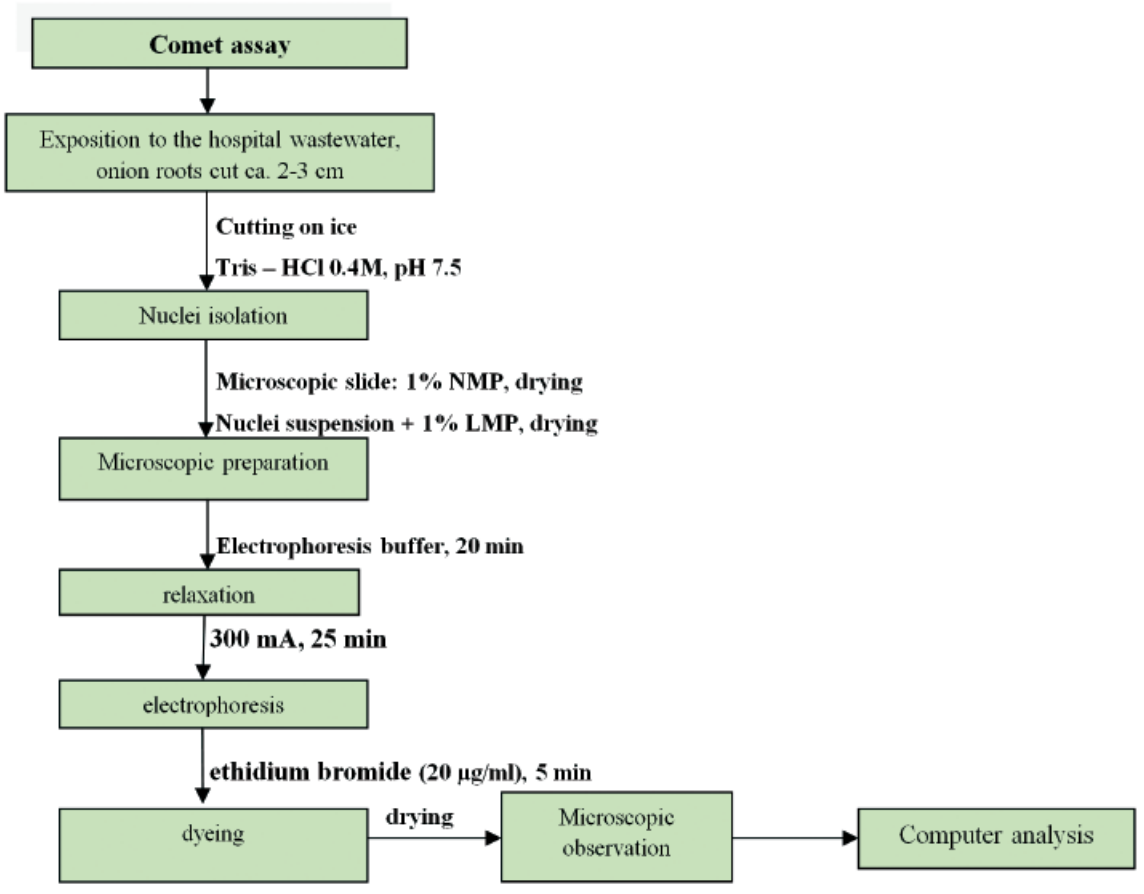


**Figure 1.**
**The comet assay scheme of the optimized procedure**

**Table 1.**
**The comparison of nuclei isolation from *A. cepa* root meristem cells in four compared protocols**

| No. | step | protocols | | | |
|---|---|---|---|---|---|
| | | [4] | [6] | [11] | [12] |
| 1. | roots cutting | no additional activity | rinsed with distilled water | placed on ice for 2 minutes | |
| 2. | nuclei isolation | roots immersed in 400 µl cold 0.4 M Tris – HCl buffer (pH = 7.5) on Petri dish, kept on ice | on Petri dish kept on ice, roots were sliced | roots immersed in 400 µl cold 0.4 M Tris – HCl buffer (pH = 7.5) on Petri dish, kept on ice | Roots immersed in 300 µl cold 0.4 M Tris – HCl buffer (pH = 7.5) on Petri dish, kept on ice |
| | | roots sliced and rubbed | nuclei suspension added to 70 µl 1 × PBS (pH=7.4) | roots sliced gently | |
| 3. | storage | suspension placed in Eppendorf tube and rub again | Nuclei : buffer suspension placed in Eppendorf tube | | |
| | | Nuclei : buffer suspension placed in Eppendorf tube, kept on ice | | | |

**Table 2.**
**The comparison of microscopic slides preparation for *A. cepa* root meristem cells in four compared protocols**

| No. | step | protocols | | | |
|---|---|---|---|---|---|
| | | [4] | [6] | [11] | [12] |
| 1. | Slides degreasing | Yes | | | |
| 2. | Slides covering with NMP agarose | 1% NMP in 1 × PBS and dried at room temperature | | 1% NMP in dH2O and dried overnight at room temperature | |
| | | 150 µl nuclei suspension and 150 µl 1% LMP in 1×PBS, covered with slides, placed on ice for ca.10 min, cover slides removed | 100 µl nuclei suspension and 100 µl 0.66% LMP in 1×PBS covered with slides, placed on ice, cover slides removed after set | mixed 40 µl nuclei suspension and 40 µl 1% LMP in 1× PBS covered with slides, placed on ice, cover slides removed after set | 150 µl nuclei suspension and 150 µl 1% LMP in 1×PBS covered with slides, placed on ice for ca. 10 min, cover slides removed |
| | | 200 µl 0.5% LMP in 1×PBS, dried for 5 min | 100 µl 0.66% LMP in 1×PBS | 80 µl 0.5% LMP in 1×PBS, dried for 5 min | |
| 3. | | placed in electrophoretic tank | | | |

**Table 3.**
**The comparison of relaxation and electrophoresis for comet assay for *A. cepa* root meristem cells in four compared protocols**

| No. | Step | protocols | | | |
|---|---|---|---|---|---|
| | | [4] | [6] | [11] | [12] |
| 1. | slides incubation on ice | no | yes | | |
| 2. | electrophoresis and buffer temperature | buffer kept in 4°C for a longer time | freshly prepared, kept in 4°C | | |
| 3. | relaxation | 15 min | 20 min | 15 min | |
| 4. | electrophoresis | 300 mA, 20 min | 300 mA, 25 min | 300 mA, 20 min | |
| 5. | washing with Tris – HCl, buffer, pH=7.5 | yes, three times | yes, for 5 min, washed with $dH_2O$ twice | yes, three times | |

potential explanation of the failures. The optimized methods is the closest to the protocol presented by [6]. Figure 1 presents the procedure of comet assay performed in the study as a result of the four protocols comparison.

#### 2.2.1. Nuclei isolation

After the cultivation of *A. cepa* in hospital wastewater the roots were cut off to obtain samples 2.5-3 cm in length, rinsed with distilled water and dried with paper towel. The roots were placed for 2 minutes on ice and immersed in 400 µl cold 0.4 M Tris – HCl buffer (pH = 7.5) on Petri dish kept on ice. The roots were gently sliced. The roots: buffer suspension was placed in Eppendorf tubes on ice immediately after cutting.

This part of the protocol seems to be similar for all the procedures. In Table 1 the comparison of this step in 4 protocols is presented (Tris – HCl buffer composition is the same).

#### 2.2.2. Microscopic slides preparation

Degreased microscopic slides were covered with 1% agarose (NMP in distilled water, Promega; w:v). Slides were dried at room temperature. In the next step 150 µl of the suspension of nuclei in 150 µl of 1% agarose (LMP in 1×PBS, Promega; w:v) in Tris – HCl buffer was placed on the slides with the first layer of agarose. The preparations were covered with cover-slides and kept on ice until set. After ca. 15 min. of incubation the cover-slides were removed and the preparations were placed in electrophoresis tank. This part of the protocol is compared in the Table 2 for 4 protocols studied.

#### 2.2.3. Relaxation and horizontal electrophoresis

Relaxation and horizontal electrophoresis were performed on ice (4°C) in electrophoresis tank (BioRad) filled with freshly prepared cold electrophoresis buffer (1 mM $Na_2EDTA$, 300 mM NaOH, pH > 13).

For DNA development microscopic slides were incubated in the buffer for 20 minutes and the electrophoresis was performed for 25 minutes at 27 V, 300 mA. The slides were washed three times with 400 mM Tris – HCl buffer (pH = 7.5). Table 3 presents relaxation and electrophoresis step in four compared protocols. The electrophoretic buffer used in the procedures was the same.

#### 2.2.4. DNA preparations dying and visualization

For DNA dying 80 µl of ethidium bromide (20 µg/ml) for each slide was used. After 5 minutes preparations were rinsed with ice-cold distilled water. Cover slides were added and they were left at room temperature to dry out. The preparations were kept in 4°C until the analysis.

The visualization of DNA damages were performed with epifluorescence microscope (MOTIC BA400T) with MOTICam Pro. 8 camera in 100×100 magnitude. Comet identification was performed with Cy3 filter, where the maximum absorbance and emission is between 550-615 nm. This step of the procedure was the same for all protocols compared.

Comet pictures were analyzed with Motic Images Plus 2.0 software for all tested media as well as for positive and negative controls.

## 3. RESULTS

First of all the comet assay was performed as it was previously presented in protocol [4]. Figure 2 presents the results of the procedure observed under the microscope. There was no presence of comets and nuclei on the slides taken into examination. Probably nuclei were not isolated and the artifacts and microorganisms were fluorescently colored in the picture.

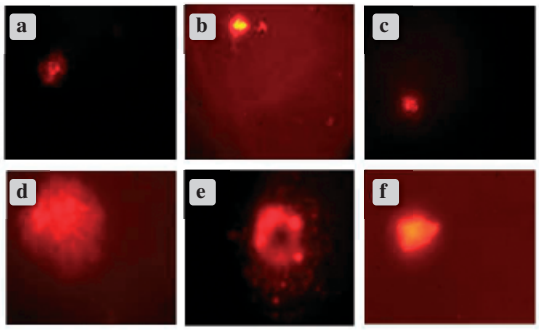


**Figure 2.**
**Photos of the slides prepared according to the SCGE procedure [4]; a-c: *A. cepa* meristem cells in negative control (water), d-f: *A. cepa* meristem cells in positive control (100% of wastewater)**

Figure 3 presents the photos of the slides prepared according to protocol [12]. A large number and diversity of the artifacts is present on these slides. Some objects formed comet-like shapes, some of them nuclei-like shapes. But it was doubtful that these elements were the comets/nuclei searched in the material. As in case of the protocol [4] the quality of the pictures obtain in this procedure was poor.

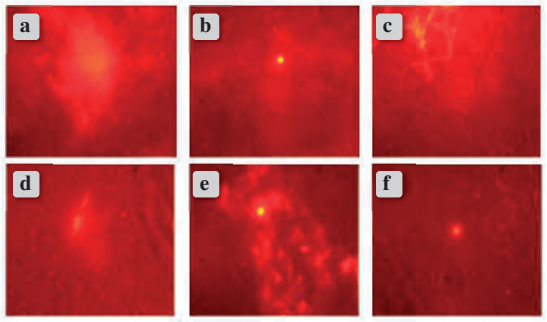


**Figure 3.**
**Photos of the slides prepared according to the SCGE procedure of [12]; a-c: *A. cepa* meristem cells in negative control (water), d-f: *A. cepa* meristem cells in positive control (100% of wastewater)**

The protocol [11] enabled to obtain cell nuclei in the isolation procedure. Nuclei membranes were blurry and some of the nuclei were poorly dyed. More objects in comet-like shapes were observed but their visualization was better than in case of two other protocols. Nonetheless, still large number of artifacts occurred in the preparations. Figure 4 presents the result of performing protocol [11] comet assay protocol for *Allium cepa* root meristem cells.

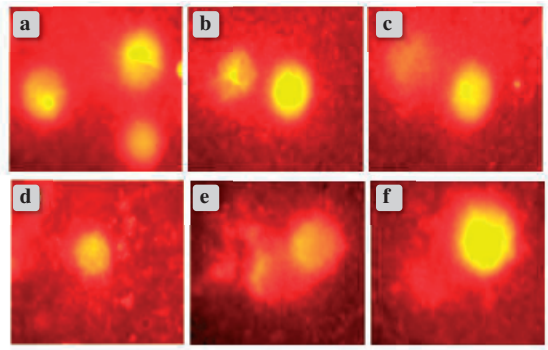


**Figure 4.**
**Photos of the slides prepared according to the SCGE procedure of [11]; a-c: *A. cepa* meristem cells in negative control (water), d-f: *A. cepa* meristem cells in positive control (100% of wastewater)**

As in protocol [11], protocol [6] presented similar procedure of comet assay and the result obtained there seemed to be the best in comet assay analysis of *A. cepa* root meristem cells. The pictures of microscopic slides obtained in the last protocol are presented (Figure 5). Both, nuclei and comets pictures were obtained. But the problem was that in one dilution of growing medium comets with a large tail, as well as undestroyed nuclei were present. The shapes of the tails were also unsatisfactory for this material. Nonetheless, the result obtained in protocol [6] gave the best results in comparison with the other three tests.

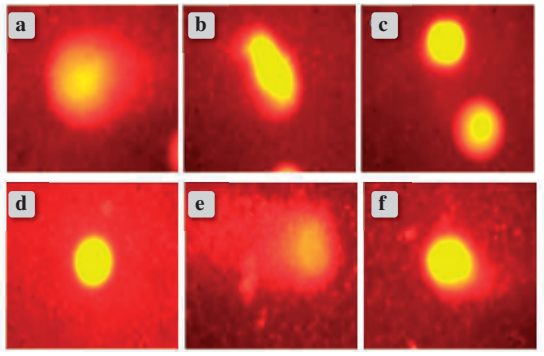


**Figure 5.**
**Photos of the slides prepared according to the SCGE procedure [6]; a-c: *A. cepa* meristem cells in negative control (water), d-f: *A. cepa* meristem cells in positive control (100% of wastewater)**

We compared the methods and the results obtained with four protocols of SCGE and the result of the experiment was not satisfactory enough. The unified, optimized protocol of comet assay for *Allium cepa* root meristem cells for this experiment was prepared on the basis of the existing protocols as their combination. The procedure of SCGE gave the satisfactory result, as it is shown in the Figure 6. The results of the test were repetitive. The comets and the nuclei shapes in the microscopic preparations were well defined, easy to recognize and with no additional artifacts. The number of nuclei obtained in the slides was high enough to find enough the objects to the proper analysis.

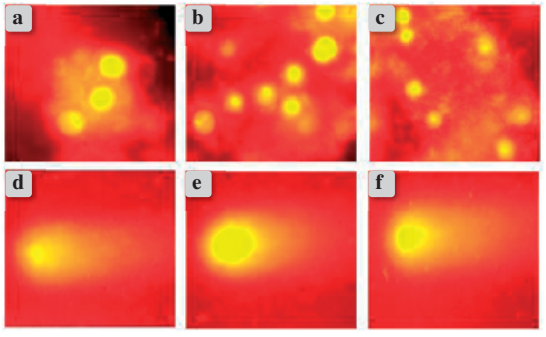


**Figure 6.**
**Photos of the slides prepared according to the optimized SCGE procedure obtained on the basis of four compared protocols; a-c meristem cells after incubation on water, d-f meristem cells after incubation on 100% of wastewater**

## 4. DISCUSSION

It is suspected that the first step of SCGE method – nuclei isolation, is crucial for the successful test performance. In optimized protocol roots after being cut off were washed with distilled water and placed on ice, as in [12] and [6] protocols. It seems that the low temperature in essential for proper tissue preparation in *A. cepa* tests. Additionally, in procedure [6] it was stated that for nuclei isolation Tris – HCl buffer (pH=7.5) should be cold. On this basis we suspected that for proper nuclei isolation for *A. cepa* not only the buffer used, but also the environment of the procedure should be cold, so this part of the protocol was performed on ice. The roots were also sliced gently in order not to destroy the tissue mechanically, unlike in the method from [4] where the roots were rubbed. The nuclei should be extracted to Tris – HCl buffer so it should be noticed that there is high possibility that the contact of the cells with the buffer is important for successful procedure. In the optimized procedure we cut the roots in constant contact with the buffer, on ice, that enabled to obtain positive result. It is not excluded that the volume of the buffer added was also important. Four hundred µl of Tris – HCl buffer per ca. 5-6 roots (2.5-3 cm in length) was enough. In all procedures the suspension of nuclei in the buffer was kept on ice. Such information point that this fact seems to be important.

The step of microscopic slides preparation depended on the proper nuclei isolation. Probably the first covering the slides with NMP agarose is not essential. Agarose in this step tend to crease but it was checked that only a part of the slide need to be covered with the agarose layer. NMP agarose can be prepared as water, not buffer solution, as it was stated in the unify protocol with no negative results to the procedure's effect. The previous protocols, [4] and [12] used NMP agarose solution in 1×PBS. Although this agarose solution covered the slides evenly no (positive or negative) result of the SCGE procedure was observed. As it is presented in the optimized procedure this step seems to be not very relevant for total procedure success.

Procedures [11] and [6] dried the NMP slides covered with agarose at room temperature overnight. We tried to repeat this step, but it appeared the agarose dry out in overnight incubation and it was difficult to apply the next agarose layers. In optimize procedure we covered the slides with agarose layer after layer directly at the day of performing the next steps of the procedure.

Nuclei suspension application in Tris – HCl buffer and LMP agarose was performed as in protocol [6]. Special attention was paid to the mixing of the nuclei suspension with LMP agarose, because a large volume of the mixture was flowing down from the slide if it was not bind with LMP agarose. This causes problems with slides microscopic observation. Because 1% agarose is soft we could prolonged the time of LMP agarose solidification on ice. The cover slides removed after this step didn't destroy the agarose surface which happen with the shorter solidification times. There was no need for adding the extra layer of nuclei-agarose suspension, as it was in protocols [4], [12] and [11].

The relaxation and electrophoresis steps depended on the electrophoretic buffer. It was noticed that the freshness and temperature of electrophoretic buffer is relevant for the procedure. In comet assay according to [4] the buffer was cooled, but prepared a few days earlier and already used. Additionally, the electrophoretic tank was placed on ice. This counteracted the buffer warming up, appearing with long electrophoresis time. In the modified protocol the buffer was freshly prepared, cooled and the environment of the electrophoresis was cooled down by placing the tank on ice. It is possible that, as it was stated [10], that the equipment for the procedure has the influence on the test sensitivity. The modification in the step of relaxation and electrophoresis were performed on the basis of two protocols: [11] and [6], which gave the best results from four protocols tested. The time of relaxation was adjusted experimentally to 20 minutes, the electrophoresis was performed for 25 minutes to obtained better resolution and sensitivity of the tests in comparison to the results obtained in the other procedures tested. This suggests also that the electrophoresis equipment has influence on the SCEG performance. The intensity of the current was 300 mA as suggested in all protocols tested.

The same technique of fluorescent dying gave different intensities of the samples. It could be caused by uneven ethidium bromide coverage of the slides or its flow down. Nonetheless the comets and the nuclei were well visible.

## 5. CONCLUSION

It was stated that although the compared procedures were similar the details of the protocols can cause problems in obtaining positive result of the test on *Allium cepa* roots meristem cells. The methods from

the bibliography shouldn't be used uncritically and with no optimization to the material used in the experiment. Probably of the genotoxic compound or test plant is different than that presented in this work the SCGE protocol should be optimize again. It should be also underline that comet assay requires precision and accuracy in each step. Probably the freshness and temperature of the solution used as well as performing the procedure on ice are crucial in proper procedure performance. Nonetheless the methods obtained from the bibliography are useful basis for optimization of the protocol for particular type of material, pollutant and equipment used. The unify protocol presented in this study can be easily repeated for the same sort of the wastewater as potentially genotoxic using the same type of the indicator – *Allium cepa* – which appeared to be useful in ecotoxicological risk assessment.

## ACKNOWLEDGEMENT

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