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Staining of protonemal cells of *Ceratodon purpureus* (Hedw.) Brid. for better highlighting micronuclei in bryophytes

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Abstract – The aim of this study is to develop a technique of visualization and staining of nuclei of protonemal cells of *Ceratodon purpureus* (Hedw.) Brid. in order to establish a test of genotoxicity (micronucleus assay). This technique is part of the development of methodology for environmental risk assessment. Bryophytes are often used as biological models in biomonitoring and ecotoxicology. For ecotoxicology studies, there are bioassays, including micronucleus assay. The genotoxicity assay is often practiced in higher plants; we wanted to expand its use to lower plants, like mosses and especially *Ceratodon purpureus*. To meet the criteria of micronucleus assay, the cells of protonemata of *Ceratodon purpureus* were selected as a model. The liquid culture of protonemata provides the quantity of biomass needed to achieve a bioassay. First, the development of a reproducible method for visualization and staining of the nuclei of protonemal cells was a necessary and prior step to the realization of micronucleus assay. So for this staining stage, the coloring agent used was the acetic orcein. It is the reference coloring agent for the micronucleus test on *Vicia faba* (AFNOR, 2004). With our assays of staining, we could develop a reproducible technique of staining of protonemal nuclei. Then, to attempt to visualize micronuclei, we used our method of staining after an exposure of protonemata to cadmium. The first test of staining of micronuclei following exposure of protonemata to Cd failed to demonstrate micronuclei in the conditions of the experiment. Further research must be conducted using other clastogenic agents to validate our results. Assays on micronuclei of cells of protonemata of *Ceratodon purpureus* are a first in scientific research. Its innovative aspect encourages further research.

Protonema / culture of protonema / coloration of nucleus of protonema / micronucleus assay

Résumé – L'objectif de cette étude est de développer une technique de visualisation et de coloration de noyaux de cellules protonématales de *Ceratodon purpureus* (Hedw.) Brid. dans le but de développer un test de génotoxicité. Cette technique s'inscrit dans le développement de méthode d'évaluation des risques. Les bryophytes sont déjà très utilisées comme modèles biologiques en biosurveillance et en écotoxicologie. Pour les études

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d'écotoxicologie, il existe des bioessais, notamment le test micronoyaux. Ce test de génotoxicité est déjà pratiqué chez les plantes supérieures, nous avons souhaité élargir son utilisation aux plantes inférieures comme les mousses et plus particulièrement *Ceratodon purpureus*. Pour répondre aux critères du test micronoyaux, les cellules protonématales de *Ceratodon purpureus* ont été choisies comme modèle d'étude. La culture en milieu liquide de protonéma permet d'obtenir la quantité de biomasse nécessaire à la réalisation d'un bioessai. La mise au point d'une méthode reproductible de visualisation et de coloration des noyaux des cellules protonématales a été une étape indispensable et préalable à la réalisation du test micronoyaux. Ainsi pour cette étape de coloration, le colorant utilisé est l'orcéine acétique. Il s'agit du colorant de référence pour le test micronoyaux sur *Vicia faba* (AFNOR, 2004). Nos essais de coloration nous ont permis de mettre au point une technique reproductible de coloration des noyaux de cellules protonématales. Ensuite, pour tenter de visualiser des micronoyaux, nous avons utilisé notre méthode de coloration après une exposition des protonémas au cadmium. Le premier essai de coloration des micronoyaux suite à une exposition de protonéma au Cd n'a pas permis de mettre en évidence de micronoyaux en l'état de l'expérience. Des recherches supplémentaires méritent d'être menées en utilisant notamment d'autres agents clastogènes afin de valider nos résultats. Ainsi, les tests micronoyaux sur les cellules protonématales de *Ceratodon purpureus* sont une première dans la recherche scientifique. Son aspect novateur encourage à la poursuite des recherches.

Protonéma / culture de protonéma / coloration des noyaux de protonéma / test micronoyaux

INTRODUCTION

The effect of pollutants could be evaluated by different methods as biomonitoring and bioassays used in ecotoxicology. And specific organisms can serve as models for these different methods. The "biomonitoring approach" may be defined as the use of bio-organisms/materials to obtain information on certain characteristics of the biosphere and to estimate the effects of pollution (Wolterbeek, 2002; Meyer *et al.*, 2010). This biomonitoring approach is complementary method to the usual chemical analysis. And, to evaluate the toxicity of pollutants exists another method using a biological model: the bioassay developed in ecotoxicology. Moreover, bryophytes, particularly mosses, are biological models often used in biomonitoring and in ecotoxicology (Denayer, 2000; Faburé *et al.*, 2010). For the study of ecotoxicology, there are many assays for the detection of cytogenetic damage after exposure to certain pollutants. The micronucleus assay is one of the most convenient techniques for the investigation of chromosome breaking effects in DNA on the cell nucleus following exposure to air pollution (Vasilenko & Sidorenko, 1995). The mitotic root meristems of *Allium cepa* and *Vicia faba* have been the pioneer cytogenetic materials for clastogenicity studies of physical and chemical agents since the early 1930s (Ma *et al.*, 1995). This bioassay was normalized in 2004 with *Vicia faba* as a biological model (AFNOR, 2004). The aim of this micronucleus assay consists of putting the presence of micronuclei in an obvious place following exposure of the biological material to a clastogenic or aneugenic agent. So, the potential use of micronucleus assays in plants for the detection of genotoxic effects of pollutants was investigated (Steinkellner *et al.*, 1998). A number of plants have been used in assays for genetic damage expressed as micronuclei after pollutant exposure but never mosses. So, it should be interesting to enhance the panel of micronucleus assay with the use of mosses and especially protonemata.

Mosses serve to assess the deposition of atmospheric pollutants. Indeed, mosses have little or no cuticle and no root, and owing to their ectohydric nature, they obtain most elements and nutrients directly from dry and bulk atmospheric deposition (Faburé *et al.*, 2010). For this study, we decided to work with an acrocarpous moss: *Ceratodon purpureus* (Fig. 1). The standardized culture of *Ceratodon purpureus* is controlled (Faburé *et al.*, 2010). This moss grows in different regions of the globe. It is an abundant cosmopolitan species often used for monitoring of air quality. Finally, this species is tolerant of environmental pollution (Smith, 1978).

Nevertheless, the micronucleus assay needs specific parameters as the staining of the nucleus and mitotic index determined. To validate micronucleus assay, we must count the number of nuclei, the numbers of micronuclei and the number of mitotic divisions, requiring a high mitotic division rate. The protonema is made up of undifferentiated cells and cells with a high mitotic division rate. We chose to work with the protonema of *Ceratodon purpureus*, obtained from the development of spores of laboratory plants. The protonema is an elongate, thread-like structure that develops from the germinated spore of mosses. The moss protonema typically branches (Fig. 2) and can develop into chloronema, caulonema, or rhizoids, depending on the species and the conditions. The chloronema is the first thread formed by the germinating spore and is distinguished by its perpendicular cross walls and short cells. The caulonema, when present, develops later and is the source of gametophore buds in those species with both types of protonemal segments. It is distinguished by its distal position relative to the spore and longer cells with oblique cross walls. The rhizoids are distinguished by their pigmented (usually brown) cell walls and oblique cross walls (Glime, 2007a).

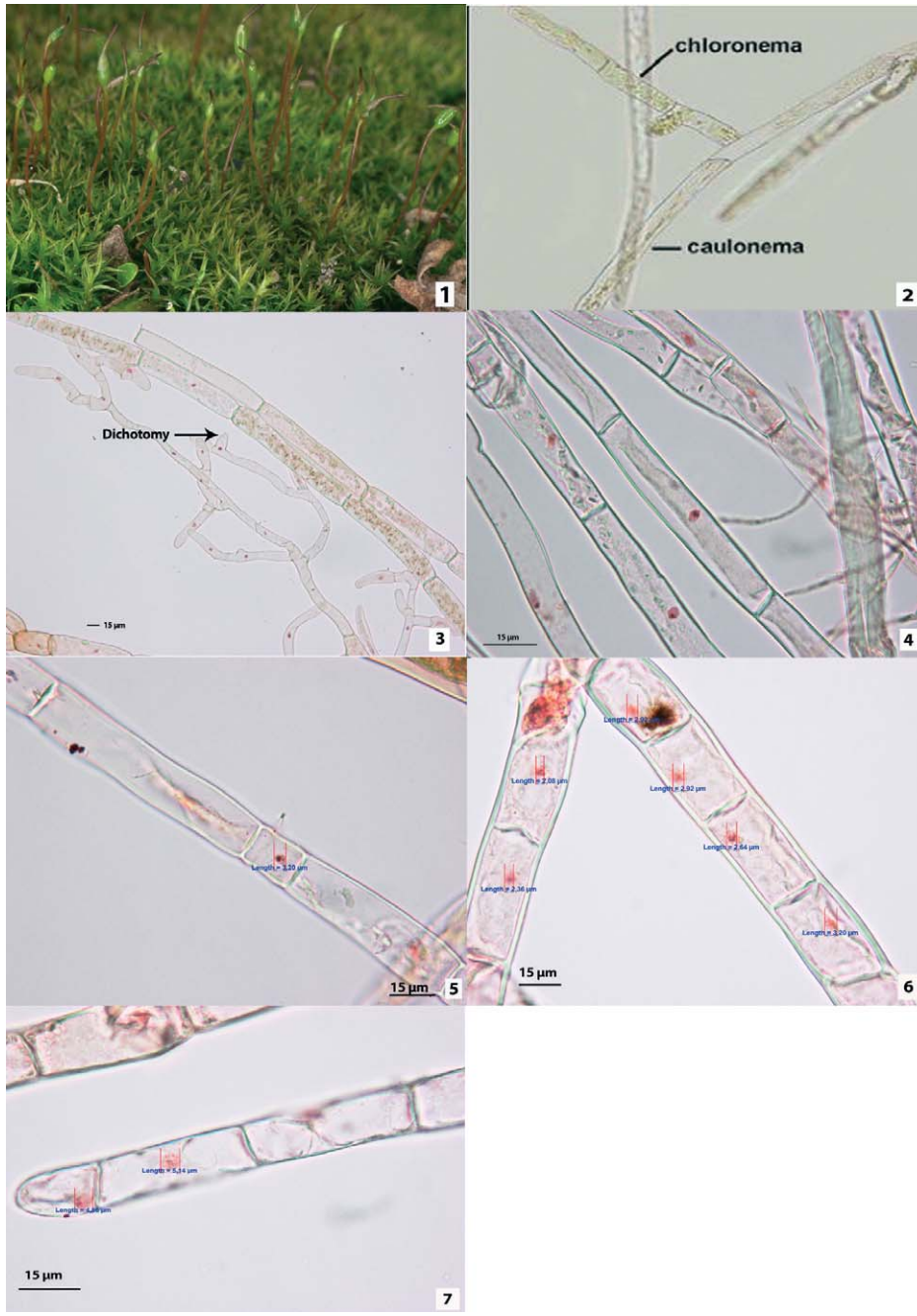
Thus, our cultures of protonemata must not cause the formation of buds because it is necessary to work with cells with a high mitotic division rate like cells of protonemata. Cultures in liquid medium should prevent the development of buds (Becquerel, 1906; Servettaz, 1913; Brown, 1919).

The objective of this study was to develop, for the first time, a process of visualization and a technique of staining of nuclei of protonemata. We wanted to see if we could use our process of staining of protonemal nuclei to develop a micronucleus assay with the protonema of *Ceratodon purpureus*. Moreover, we wanted to evaluate if the protonemal tissue of *Ceratodon purpureus* can be used as a biological model for a genetoxicity assay with an approach to environmental risk assessment. Thus, an exposure to cadmium was realized to see if the staining technique allowed visualizing micronuclei.

MATERIALS AND METHODS

The culture medium

We used 60 ml culture tubes with closer-caps and a diameter of 35 mm, height of 70 mm filled up with a liquid medium of Kofler Medium A solution (Kofler, 1959) (Tab. 1) completed with a Heller oligodynamic solution (Tab. 2). The final nutritive solution of Kofler A with Heller oligodynamic solution consists of 100 mL of Kofler Medium A solution concentrated by a factor of 10 without Cl_3 Fe, $6H_2O$ (Tab. 1), with 0.1 mL of Heller oligodynamic solution (Tab. 2), and with 899.9 mL of distilled water.



Figs 1-7. **1.** *Ceratodon purpureus* (Hedw.) Brid. (Photo by Malcolm Storey). **2.** Distinction of chloronema and caulonema on the protonema of *Funaria hygrometrica* (Photo by Janice Glime). **3-6.** Nuclei of cells of protonemata of *Ceratodon purpureus* (Photo by Ludivine Canivet, 2008). **7.** Cells of protonemata exposed at a concentration of 10^{-3}M of cadmium (Photo by Ludivine Canivet, 2008).

Table 1. Composition of Kofler Medium A solution (Kofler, 1959)

Medium A	*Concentration (g/L)*
$NO_3 NH_4$	0,5
$(NO_3)_2 Ca, 4H_2O$	0,25
$SO_4 K_2$	0,25
$SO_4 Mg, 7H_2O$	0,25
$PO_4 H_2 K$	0,20
$PO_4 H Na_2, 2H_2O$	0,05
$Cl_3 Fe, 6H_2O$	0,001
Eléments oligodynamiques	+

Table 2. Composition of Heller oligodynamic solution (Kofler, 1959)

Heller oligodynamic Solution	*Concentration (mg/L)*
$Cl_3 Fe, 6H_2O$	1
$SO_4 Zn, 7H_2O$	1
$BO_3 H_3$	1
$SO_4 Mn, H_2O$	0,1
$SO_4 Cu, 5H_2O$	0,03
IK	0,01

Production of protonema of Ceratodon purpureus

The spores used in our process were mature spores from one capsule of *Ceratodon purpureus* collected from the site of an industrial wasteland located in Don (Northern France).

Ceratodon purpureus was cultivated in controlled conditions described in the following section.

Before inoculation, all equipment was cleaned with household sodium hypochlorite bleach (5%) and then with ethanol (95%). The culture medium was sterilized by autoclaving at 121°C for 21 min. The spores contained in the capsule were inoculated in the nutritive solution under a laminar flow-hood. The capsule was sterilized by successive baths: in ethanol (1 min) and in the household sodium hypochlorite bleach (1 min). A rinsing in ethanol was made for several seconds. One capsule was used for experimentation to reduce genetic heterogeneity, interesting for the micronucleus assay. The capsule was opened in a watch glass containing the nutritive solution. Then, 20 µL of nutritive solution, containing spores was placed in a culture tube containing 45 ml of nutritive solution. The culture tubes were closed and surrounded by parafilm. For each culture, five replicates were put in a culture chamber (Phytobac®, Strader).

An optimal temperature of 22 ± 2°C (Glime, 2007a) was applied, and a white light was used (Glime, 2007a). The culture chamber was equipped with fluorescent light F58 Activa 172 day light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The presence of liquid water is also essential (Glime, 2007b). Moreover, it is recommended to alternate day/night, with 16 hours of day and with 8 hours of night in the same conditions of temperature. A continuous light accelerates senescence and reduces the chlorophyll content of mosses (Valanne, 1977).

To maintain a certain level of genetic homogeneity and to increase the biomass of protonemata, we chose to transplant the protonemata in new culture tubes. During this step, we kept the same conditions of sterilization. The protonema tissue of a culture tube was separated after 21 days of culture into 6 parts under laminar flow hood using ultrafine forceps. And always in the laminar flow hood, each part of protonema was placed into a new culture tube containing 45 ml of nutritive solution diluted by a factor 10 to avoid the toxicity of ammonium nitrate present in the nutritive solution. However, the ammonium nitrate is necessary for the spore's germination (Kofler, 1959).

The staining of nuclei of protonemal cells

The coloring agent used to highlight the nuclei and therefore the DNA is orcein 1% diluted in acetic acid 45%. This mixture was brought to a boil for 10 min, then filtered after cooling. It is important to filter the staining agent before each use to prevent the formation of crystals of orcein. These crystals could be mistaken for micronuclei during microscopic examination of cells (AFNOR, 2004).

First, all filaments of protonemata were retrieved with high-precision stainless forceps. These filaments were fixed by immersion in Carnoy's solution overnight at 4°C (Bernie, 1966; Jovet-Ast, 1969; AFNOR, 2004). Carnoy's solution is composed of glacial acetic acid and ethanol in proportions of 25% and 75%. This solution must be freshly prepared (AFNOR, 2004).

A coloring agent process has been developed specifically for the protonemata of bryophytes. Some filaments were retrieved with high precision and were placed in distilled water for 5 min. Then, some filaments were deposited on a slide and separated under a binocular microscope using ultrafine forceps. A binocular microscope (WILD M3) (magnification ×400) was used to allow a better homogeneity of filaments of protonemata on the slide.

Afterwards, we added a drop of orcein for 5 min and the preparation was crushed between slide and cover glass. Then, the protonema was rinsed by ethanol by capillarity between slide and cover glass. Finally, the protonema was rinsed with a drop of distilled water, always by capillarity.

Our preparation was observed under an optical microscope (Nikon, Eclipse E200) at magnification ×1000, using immersion oil (refractive index at 23°C = 1.5150 and viscosity = 150 cSt).

Exposure of protonema to cadmium

The clastogenic substance used is cadmium nitrate with a molar mass of 308.47 $g \cdot mol^{-1}$ (Acros Oganics).

After 21 days of culture, the protonema tissue grown in a culture tube was divided in 8 parts and exposed to cadmium. Two culture tubes were inoculated in nutritive solution for each concentration in Cd: control (no cadmium), 10^{-3} mM, 10^{-2} mM and 1 mM.

Knowing that the cells of mosses undergo approximately one cell division per day (Kofler, 1959), the exposure lasted three days.

Then, all protonema tissues were stained and observed as described earlier. We counted the number of nuclei, the number of mitotic divisions and the number of micronuclei.

RESULTS

We obtained two types of results with our tests. First, we developed our technique of visualization and staining of nuclei of protonemal cells of *Ceratodon purpureus*. Second, we stained the micronuclei of our protonemata exposed to cadmium.

The development of the process of staining of nuclei allowed us to visualize for the first time the nuclei of cells of protonemata of *Ceratodon purpureus* (Figs 3-4). Nuclei were recognized by their granular appearance. In

addition, nuclei were stained red with orcein acetic which was specifically attached to DNA. Moreover, we could justify that these are nuclei because we could observe one nucleus per cell.

We measured the diameter of the nucleus, showing that the size of the nucleus of cells in the protonema of *Ceratodon purpureus* is about 3 μm in diameter (Figs 5-6). The staining process development allowed us to observe approximately 550 nuclei using 5 slides.

The culture medium, the culture conditions and the transplanting allowed us to obtain a ball of protonemata that occupied between 30% and 50% of each culture tube volume (8 tubes cultures). This observation was evaluated by the naked eye by the author. The preparation of biological material needed to complete the micronucleus assay allowed us to demonstrate the effectiveness of our technique of visualization and staining of nuclei of protonemal cells. In addition the transplant system has an advantage which is to achieve a micronucleus assay with samples that are genetically similar. This genetic similarity avoids bias that might influence the formation of possible micronuclei. As regards different concentrations of Cd tested in protonemata of *Ceratodon purpureus*, the total number of nuclei observed at 10^{-3} mM and 10^{-2} mM concentrations (Tab. 3) was similar, allowing comparison of samples. However, even if the nuclei were well observable, neither micronucleus and nor cell division could be observed. At higher concentrations of Cd (1 mM), no micronucleus has been observed by the author. Then, we can note that the number of nuclei observed in the protonemata exposed to a concentration of 1 mM is lower than the number of nuclei observed in the protonema not exposed to cadmium (Tab. 3). Protonemal cells exposed to a concentration of 1 mM Cd show some cell damage which might explain why the nuclei are more difficult to observe. Indeed, the membranes of cells were altered and even broken. Moreover, the average diameter of nuclei of cells of protonemata exposed to a concentration of 1 mM of Cd increased to 4-6 μm instead of 2-3 μm (Fig. 7).

Table 3. Number of nuclei in each sample visualized at the exposition

Concentration in Cd	*Control*	*10^{-6}M*	*10^{-5}M*	*10^{-3}M*
1	48	63	102	90
2	106	40	97	78
3	97	247	56	51
4	131	82	127	65
5	171	133	155	41
Total	**553**	**565**	**537**	**325**

DISCUSSION

As regards the staining of the nuclei of protonemal cells of *Ceratodon purpureus*, it was possible to validate our method of visualization and staining of nuclei of protonemal cells using orcein-like coloring agent, with some developments specifically for the protonema. The nuclei were clearly visible. During the tests, an average of 550 nuclei was observed with 5 slides. Thus, by increasing the sample size, we can stain, view and count a sufficient number of nuclei for the realization of a micronucleus assay.

The exposure to cadmium was a test to see if our staining technique allowed visualizing micronuclei. If we want to validate the micronucleus assay on protonemal cells in the same conditions as those of the norm AFNOR (2004), we must observe mitotic division. However, no mitotic division could be observed. But there was growth of protonemata. During our observations, we saw figures showing the beginning of dichotomies resulting from the next cell division (Fig. 3). According to Kofler (1959), cell division is not observable by itself, explaining why it is difficult to visualize cell division in mosses. All prophase phenomena that occur inside the nuclear membrane are not visible with an optical microscope as chromosomes in metaphase and anaphase. However, Kofler (1959) indicates that the cells ready to divide can be found without difficulty. Thus, the size of apical cells (1.5 to 1.7 times the length of the cells behind it) and the slight decline of the nucleus relative to the apex are indications of cell division.

During exposures to Cd, the nuclei were clearly visible and consistent at concentrations of 10^{-3} mM and 10^{-2} mM cadmium or control, and they had a diameter of about 2 to 4 μm. The membrane and cell wall were not damaged. But no micronucleus was observed. Several hypotheses could explain this result. It must be remembered that a micronucleus is a fragment of a nucleus, and its size is smaller than the size of a nucleus. The size of the fragment of chromosome forming the micronucleus may be insufficient to allow microscopic observation. Indeed, the quantity of DNA present in the micronucleus was not sufficient to obtain a good contrast to allow observation with certainty. During our observations, we could see some "red dots" in the protonemal cells that could be micronuclei, but these "red dots" were evaluated as an artifact (Fig. 7). Other observations should be necessary to validate this point. Eventually, the concentrations tested, 10^{-3} mM and 10^{-2} mM, may be ineffective to cause damage at the cellular level and especially at the nuclear level because we didn't observe micronuclei as in controls.

At the concentration of 1 mM, nuclei were observed but in smaller numbers compared to the control. This result could be explained by a cytotoxic effect of cadmium as evidenced also by alteration to the plasma membrane. It is known that the cadmium crosses the membrane barrier in mosses (Bargagli, 1993). This suggests that the effects would have reached the genetic material. Recently, the only observations, that we made, were an increase of nuclear diameter of 4-6 μm. Other authors have observed distortion of nuclei but we didn't observe this phenomenon in our observations. Others have noted that cadmium has an effect on the shape of the nucleus. Hala *et al.* (2007) have observed abnormalities on nuclei in interphase in *Allium cepa* and *Vicia faba* due to contamination by industrial effluents and also causing micronuclei, which demonstrate the genotoxic effects of industrial effluents. Similar effects have been observed in *Raphanus sativus*, var Redondo Vermelho. Following exposure to 1 mM cadmium, Vitória *et al.* (2006) observed an intense degradation and disruption of the nuclear membrane. Similarly, Kuthanová *et al.* (2004) observed a change in shape of the nucleus in *Nicotiana tabacum* L. The nucleus tended to elongate after treatment with 0.05 mM of cadmium.

Thus, we could observe an increase of the nuclear diameter of protonemal cells after an exposure to cadmium. This does not exclude the formation of micronuclei, but they were not observed in our experiment. Finally, it would be interesting to do further research to work on the visualization of micronuclei and mitotic divisions. Similarly, exposure should be tested with another clastogenic agent, such as hydrazine maleic, reference substance in the AFNOR norm (2004).

CONCLUSION

Bryophytes are very interesting organisms in research of biological models in ecotoxicological integration in approach to environmental risk assessment. In addition, our laboratories want to develop biomarkers of genotoxicity in order to complete the list of bioassays known and used to assess the toxicity of pollutants. Mosses have shown their effectiveness in accumulating pollution. The development of bioassays on lower plants, as mosses, is part of an innovative approach. The development of technique of visualization and staining of protonemal cells to observe nuclei was realized in a reproducible manner. This staining method also allows visualizing and counting a sufficient number of nuclei for the realization of ecotoxicological assays. Although early exposures are not given, convincing results with exposition to cadmium as a clastogenic agent, it would be interesting to do further research using other clastogenic agents such as hydrazine maleic. Thus, despite the lack of observation of micronuclei, the visualization of cell nuclei of protonemata of *Ceratodon purpureus* is a first in the study of bryophytes. Moreover, there are few studies on air pollution; these tests could be adjusted for air. These investigations are part of a highly innovative step. The research of micronuclei on cells of protonemata of bryophytes is a first in scientific research on mosses and bryophytes. In the future, this assay should be usable as a regulatory test to evaluate the impact of atmospheric pollutants and assess the new chemical substance risks.

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REFERENCES

AFNOR, 2004 — Qualité du sol, Evaluation des effets génotoxiques sur les végétaux supérieurs, NF T 90-327. *AFNOR*, 13 p.

BECQUEREL P., 1906 — Germination des spores et morphologie des protonémas d'*Atrichum undulatum* et d'*Hypnum velutinum* en milieux stérilisés. *Revue générale de botanique* 18: 49-68.

BERNIE G.K., 1966 — Polyploïdy in some West African species of Riccardia (Gray). *Revue bryologique et lichénologique* 34(1-2): 302-308.

BARGAGLI R., 1993 — Plant leaves and lichens as biomonitors of natural and anthropogenic emissions of mercury. *In:* Markert B. (ed.), *Plants as biomonitors indicators for heavy metals in the terrestrial environment*. Weinheim: VCH Publisher, pp. 461-484.

BROWN M. M., 1919 — The development of the gametophyte and the distribution of sexual characters in *Funaria hygrometrica*. *American journal of botany* 6: 387-400.

DENAYER F.-O., 2000 — Ecotoxicité des éléments traces métalliques chez les bryophytes. Mise au point d'un bryocapteur des retombées atmosphériques à l'aide de *Ceratodon purpureus* (Hedw.) Brid. Thèse pour l'obtention de grade de docteur de l'Université de Metz. Metz, 238 p.

FABURE J., MEYER C., DENAYER F, GAUDRY A., GILBERT D. & BERNARD N., 2010 — Accumulation capacities of particulate matter in an acrocarpous and a pleucarpous moss exposed at three differently polluted sites (industrial, urban and rural). *Water, air and soil pollution*, DOI 10.1007/s11270-010-0333-0.

GLIME J. M., 2007a — Ecophysiology of development: protonema. *In: Bryophyte Ecology*, Volume 1, Physiological Ecology, Ebook sponsored by Michigan Technological University and

the International Association of Bryologists, accessed on June 5, 2010 at <http://www.bryoecol.mtu.edu/>.

GLIME J. M., 2007b — Ecophysiology of development: spore germination. *In: Bryophyte Ecology*, Volume 1, Physiological Ecology, Ebook sponsored by Michigan Technological University and the International Association of Bryologists, accessed on June 5, 2010 at <http://www.bryoecol.mtu.edu/>.

HALA M., YEHIA A. & WAEEL M., 2007 — Use of plant genotoxicity bioassay for the evaluation of efficiency of algal biofilters in bioremediation of toxic industrial effluent. *Ecotoxicology and environmental safety* 66: 57-64.

JOVET-AST J., 1969 — Le caryotype des Ricciaceae. *Revue bryologique et lichénologique* 36 (3-4): 673-689.

KOFLER L., 1959 — Contribution à l'étude biologique des Mousses cultivées *in vitro* : germination des spores, croissance et développement du protonéma chez *Funaria hygrometrica*. *Revue bryologique et lichénologique* 28(1-2): 202 p.

KUTHANOVÁ A., GEMPERLOVÁ L., ZELENKOVÁ S., EDER J., MACHÁČKOVÁ I., OPATRNY Z. & CVIKROVÁ M., 2004 — Cytological changes and alterations in polyamine contents induced by cadmium in tobacco BY-2 cells. *Plant physiology and biochemistry* 42: 149-156.

MA T., XU Z., XU C., McCONNELL H., RABAGO E., ARREOLA G. A. & ZHANG H., 1995 — The improved *Allium/Vicia* root tip micronucleus assay for clastogenicity of environmental pollutants. *Mutation research* 334: 185-195.

MEYER C., GILBERT D., GAUDRY A., FRANCHI M., NGUYEN-VIET H., FABURE J. & BERNARD N., 2010 — Relationship of atmospheric pollution characterized by gas (NO_2) and particles (PM10) to microbial communities living in bryophytes at three differently polluted sites (rural, urban, industrial). *Microbial ecology* 59: 324-334.

SERVETTAZ C., 1913 — Recherches expérimentales sur le développement et la nutrition des Mousses en milieu stérilisé. *Annales des sciences naturelles, Botanique,* ser. 9, 17: 111-223.

SMITH A.J.E., 1978 — The Moss Flora of Britain & Ireland. Cambridge: Press Syndicate of the University of Cambridge, 706 p.

STEINKELLNER H., MUN-SIK K., HELMA C., ECKER S., MA T., HORAK O., KUNDI M. & KNASMULLER S., 1998 — Genotoxic effects of heavy metals: comparative investigation with plants bioassays. *Environmental and molecular mutagenesis* 31: 183-191.

VALANNE N., 1977 — Effect of continuous light on CO_2 fixation, chlorophyll content, growth and chloroplast structure in *Ceratodon purpureus*. *Zeitschrift für Pflanzenphysiologie* 81: 347-357.

VASILENKO A. & SIDORENKO P. G., 1995 — Induction of micronuclei in plant cells after exposure to accelerate ion irradiation. *Radiation environmental biophysics* 34: 107-112.

VITÓRIA A.P., DA CUNHA M. & AZEVEDO R.A., 2006 — Ultrastructural changes of radish leaf exposed to cadmium. *Environmental and experimental botany* 58: 47-52.

WOLTERBEEK B., 2002 — Biomonitoring of trace element air pollution: principles, possibilities and perspectives. *Environmental pollution* 120: 11-21.