CHANGES IN DNA, DRY MASS AND PROTEIN CONTENT OF LEAF EPIDERMIS NUCLEI DURING AGING OF PERENNIAL MONOCOTYLEDONOUS PLANTS

HANNA KURAN

Department of Plant Cytology and Cytochemistry, Institute of Physiology and Cytology, University of Łódź, S. Banacha 12/16. 90-237 Łódź, Poland

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ABSTRACT

DNA, NYS and DNFB protein contents were measured cytophotometrically using the Feulgen method in the nuclei of the epidermis from the basal zone of young leaves and from the basal and apical zones of old leaves in two perennial monocotyledonous species, *Clivia miniata* and *Rhoeo discolor*. Dry mass was determined interferometrically. It was shown that nuclei with a 2C DNA content dominated in both zones of old leaves, and that a significant percentage of cells with a DNA content below 2C were present. The ratio between euchromatin DNA and heterochromatin DNA indicates a greater decrease in euchromatin during aging. Changes in DNA due to real DNA loss are accompanied by decreases in NYS and DNFB-stained proteins and a decrease in dry mass content correlated mainly with the decrease in the amount of NYS proteins.

KEY WORDS: leaf epidermis, senescence, perennial monocotyledons, DNA content, dry mass, protein content, *Clivia*, *Rhoeo*

INTRODUCTION

Numerous authors have shown that during maturation and then aging, the contents of RNA, DNA, proteins, nitrogen and chlorophyll diminish steadily in the cells of different leaf tissues of annual plants (Dhillon and Miksche 1981, Harris and Schaefer 1981, Harris et al. 1982, 1984, Thimann 1983). The decrease in the above-mentioned compounds is preceded by a decrease in the content of nucleic acids (Harris and Schaefer 1981, Harris et al. 1984, Sodmergen et al. 1989). The decline in photosynthesis observed during aging is caused by the substantial decrease in the number of chloroplasts per unit area (Kura-Hotta et al. 1990). The contents of both nucleic acids steadily decrease starting from the early stages of aging, with the DNA content decreasing more slowly than RNA (Thimann 1983). The drop in nucleic acid and protein contents leads to a reduction of nuclear dry mass (Harris et al. 1984). The nuclear DNA content is only a small fraction of the macromolecular nuclear dry mass, the main components of which are proteins (Olszewska et al. 1990).

Cytophotometric measurements of nuclear DNA content during maturation and aging of *Vicia faba* leaves show a decrease in DNA content to 2C (Olszewska et al. 1986) and a decline in the percentage of nuclei with 4C DNA content in *Zea mays* (Harris et al. 1984). Examination of the DNA level in three zones of *Pennisetum purpureum* leaves of different ages shows that the cells in the basal and middle zones of older leaves contain higher percentages of 2C nuclei than any zone in young leaves (Taylor and Vasil 1987). A substantial decrease in DNA content is observed in the epidermis and mesophyll of *Nicotiana tabacum* and *Arachis hypogea*, and a more rapid decrease in DNA content is noted in epidermal nuclei than in mesophyll nuclei (Harris et al. 1982).

The nuclei of mesophyll cells in mature *Vicia faba* leaves show an apparent decrease in nuclear DNA related to the increase in the degree of chromatin condensation (Olszewska et al. 1986). Chromocentric DNA containing condensed chromatin shows a lower sensitivity to HCl hydrolysis during the Feulgen reaction than euchromatin resulting in possible masking of C-chromatin; moreover, a considerable increase in the level of chromatin condensation is found, in mature cells although even nuclei with 4C DNA appear after longer hydrolysis (Olszewska et al. 1986). The amount of basic nuclear proteins is connected with chromatin condensation. The basic protein content in mature leaves of flowering *Vicia faba* plants is 20% higher than in root meristem cells of the same plant (Olszewska et al. 1986).

The aim of the present experiments was to examine the changes in DNA, protein and dry mass content accompanying aging of epidermal cells in perennial monocotyledonous species and determination of a leaf zone in which the cells show symptoms of aging, assessed on the basis of the above-mentioned symptoms.

MATERIAL

The experiments were carried out on the leaf epidermis of two different species of perennial monocotyledonous plants – *Clivia miniata* (*Amaryllidaceae*) and *Rhoeo discolor* (*Commelinaceae*). Five mm long parts of the epidermis were taken from the basal zone (white) of the youngest leaves and from the basal and apical zones of older leaves, whose top parts had become slightly yellow.

METHODS

Cytophotometry – Fragments of epidermis were fixed in MAF (methanol : formalin : glacial acetic acid, 80 : 15 : 5 v/v) for 24h in room temperature. The DNA contents in euchromatin and chromocenters were measured in a Zeiss histophotometer (Jena) using a 550 nm filter. The material was hydrolized before measurement for 1h, 2h and 4h in 4N HCl, at room temperature and then stained for 30 min in Schiff's

reagent prepared from pararosaniline (Gurr). The 2C and 4C DNA values were calculated from the measurements of 20 telophase and 10 prophase epidermal nuclei from the basal zone of the youngest leaves. Fifty nuclei were measured in each tested zone. The DNA content was calculated in arbitrary units (AU).

Protein content (DNFB-Feulgen and Feulgen-NYS) was determined in Feulgen-stained nuclei, first with the use of a 550 nm filter (DNA), next with a 480 nm filter (proteins) (Tas and James 1981, Marciniak and Bielecka 1986, Olszewska et al. 1987).

Interferometric measurements – Macromolecular nuclear dry mass was measured in an MPI-5 interference-polarization microscope, using squashed preparations macerated with 1N HCl and mounted in water. The material was fixed in 2% glutaraldehyde in 0.1M cacodyl buffer, pH 7.2 for 24h (Kuran and Olszewska 1977). The use of glutaraldehyde was necessary because formalin fixatives cause a 40% loss in dry mass content and a 20% decrease in nuclear size (Kufliński and Kao Yan 1980).

Determination of the presence of tannins – Due to the possible interference of tannins with the Feulgen reaction (Greilhuber 1988), a test with $K_2Cr_2O_7$ was performed. It gave negative results in all parts of the leaves studied.

RESULTS

Mitotic activity – Mitotic activity was estimated as the mitotic index (MI) in all tested leaf zones (Table 1). Cell divisions were present only in the basal zones of the youngest leaves, i.e., where the meristematic zone in the epidermis of monocotyledonous species is situated. The MI value (%) was low and identical in both tested species.

TABLE 1. Mitotic activity in epidermis of different leaf zones

Species	Young leaf basal zone	Old leaf basal zone	Old leaf apical zone
Clivia miniata	1.9±0.2	0.0	0.0
Rhoeo discolor	1.8±0.3	0.0	0.0

The effect of length of hydrolysis on Feulgen staining in the nuclei from the epidermis of the basal zone of the youngest leaf – In *Clivia miniata* the highest DNA value (AU) was observed after 2h of hydrolysis, while in *Rhoeo discolor* after 1h (Fig. 1a, b). The prolongation of hydrolysis from 2h to 4h did not change the intensity of staining of telophases and prophases. The DNA content in euchromatin and in the chromocenters of meristematic nuclei reached the highest values after the same length of hydrolysis as in prophases and telophases (Fig. 1a, b). For every tested time of hydrolysis, the DNA content in euchromatin and chromocenters increased considerably in the range of 2C-4C classes and a doubled euchromatin DNA content was observed after 2h and 4h of hydrolysis in both species. The DNA content in chromocenters doubled in each series in classes 2C-4C (Fig. 1a, b).

Nuclear DNA content in young and old leaves – The DNA content in interphase nuclei in the basal zone of the youngest leaves was 2C-4C. Regardless of the length of hydrolysis, nuclei with a 2C DNA content prevailed in *Clivia miniata* and their number remained similar after different times of hydrolysis (Fig. 2a). In *Rhoeo discolor*, longer times of hydrolysis

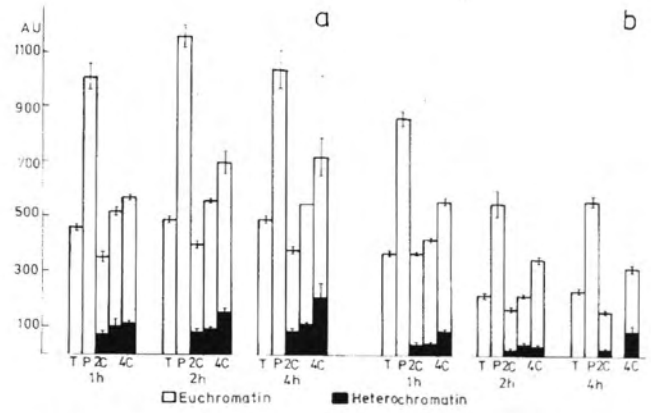

Fig. 1. The influence of duration of hydrolysis on the intensity of staining in the Feulgen procedure.
T – telophases, P – prophases, a – *Clivia miniata*, b – *Rhoeo discolor*.
Ordinate – DNA content (AU), secant – duration of hydrolysis

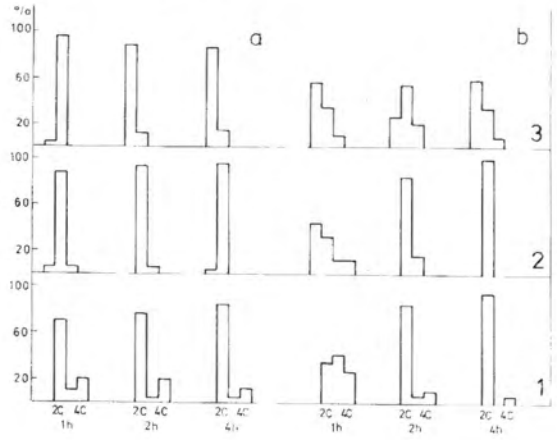

Fig. 2. DNA content of nuclei.
1 – young leaves, basal zones;
2 – old leaves, basal zones;
3 – old leaves, apical zones.
a – *Clivia miniata*,
b – *Rhoeo discolor*.
Ordinate – number of nuclei, secant – DNA content in C values after different times of hydrolysis

(2h and 4h) caused, a significant increase in the class of nuclei with 2C DNA content (Fig. 2b).

In the basal and apical zones of old leaves the number of nuclei with a lower DNA content increased. In *Clivia miniata* nuclei with 4C DNA disappeared and nuclei with content below 2C appeared. The number of these nuclei increased in the apical zone of old leaves with increasing time of hydrolysis; after 2h and 4h hydrolysis more than 86% of the nuclei belonged to that class. Similar results were obtained in *Rhoeo discolor*: after 2h hydrolysis 27% of the nuclei in the apical zone of old leaves contained less than 2C DNA, while after 4h there were 57% of such nuclei (Fig. 2a, b). This class was not as numerous in the basal zone of old leaves and dominated only in *Rhoeo discolor* after 1h hydrolysis (Fig. 2b).

The decrease of DNA content in the euchromatin nuclei from old leaves of the tested species was greatest after the optimum time of hydrolysis (2h – *Clivia*, 1h – *Rhoeo*) and reached about 50% in the apical zone (Fig. 3a, b). The chromocenter DNA of old leaves displayed a higher resistance to acid hydrolysis and its content did not decrease until 4h of hydrolysis (Fig. 3a, b). Age-related chromatin condensation was revealed by the increased DNA content in chromocenters

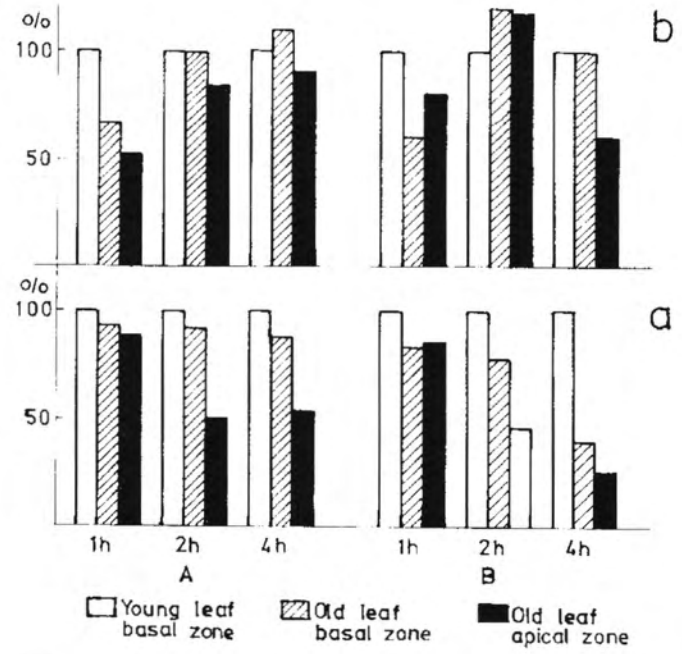

Fig. 3. DNA content in euchromatin (A) and heterochromatin (B) in nuclei of old leaves after different durations of hydrolysis.
a – *Clivia miniata*,
b – *Rhoeo discolor*.
Ordinate – number of nuclei, secant – duration of hydrolysis

after a shorter period of hydrolysis. The ratio of euchromatin DNA to heterochromatin DNA increased significantly only after 4h hydrolysis in both species, which indicates a greater loss in chromocenter DNA than in euchromatin DNA after prolongation of hydrolysis (Fig. 4a, b). *Clivia* and *Rhoeo* nuclei usually contain several chromocenters of different size. The mean chromocenter area of the meristematic zone nuclei of *Rhoeo* is half that of *Clivia* (Fig. 5a, b). After 1h and 2h of hydrolysis the chromocenter area per nucleus increased in old leaves, while after 4h hydrolysis the chromocenter size decreased (Fig. 5).

Nuclear protein content in the epidermis – The content of nuclear proteins stained with NYS and DNFB in the euchromatin in the epidermis of the youngest leaves (meristematic zone) showed a significant increase from 2C to 4C classes (Fig. 6a, b). In old leaves, the content of both kinds of euchromatin proteins decreased significantly in the oldest of the tested zones, i.e., the apical zone (Fig. 6a, b). Only in *Clivia*

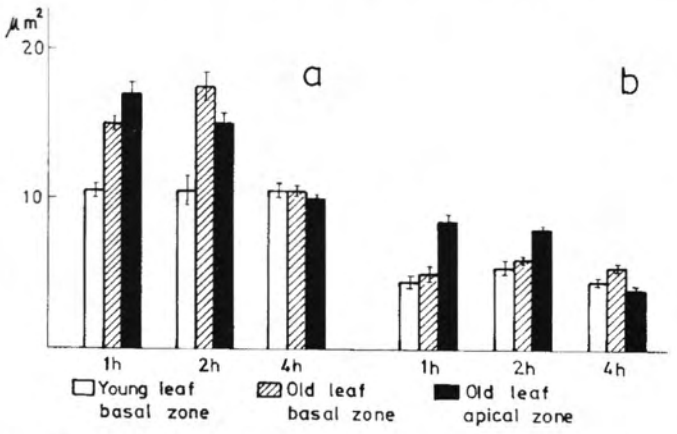

Fig. 4. Euchromatin/heterochromatin ratio after different times of hydrolysis.
a – *Clivia miniata*, b – *Rhoeo discolor*

the nuclear NYS protein level in the basal zone of old leaves was lower than in the meristematic zone (Fig. 6a), while in

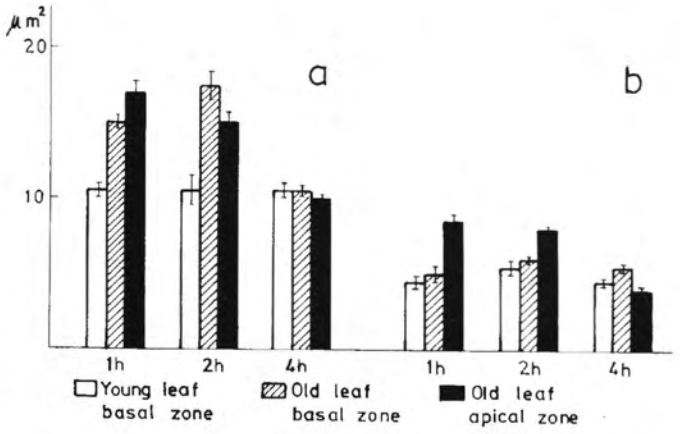

Fig. 5. Mean chromocenter area after different durations of hydrolysis.
a – *Clivia miniata*,
b – *Rhoeo discolor*.
Ordinate – surface of chromocenters, (um^2), secant – time of hydrolysis

Rhoeo the content of proteins stained with NYS and DNFB was higher than that found in meristematic zone nuclei with 2C-4C and 4C DNA contents (Fig. 6b). NYS- and DNFB-stained proteins were present in similar levels in the meristematic zone chromocenters in classes from 2C to 4C. Their content, expressed in AU, in *Rhoeo discolor*, was only half that in *Clivia miniata* (Fig. 6a, b). The chromocentric protein content in old leaves was generally lower than in young ones (Fig. 6a, b).

The ratio of DNFB protein content to DNA (Fig. 7a, b) was similar in old and young leaves of the studied species, with some decrease observed in the apical zone chromocenters of old *Rhoeo discolor* leaves. This shows that the protein loss in chromocenters of that zone was greater than their DNA loss. The ratio of NYS protein content to DNA content was different. In *Rhoeo*, the NYS protein/DNA ratio increased in the chromatin and chromocenters of old leaves (Fig. 7b), while a marked decrease in this ratio was observed in *Clivia*, especially in euchromatin. This points to a greater loss in old leaves of NYS than DNFB proteins (Fig. 7a).

Changes in dry mass content in the epidermis – The dry mass content in basal zone nuclei of young leaves of both species doubled, which corresponds to the presence of 2C, 2-4C and 4C nuclei (Fig. 8a, b). In the apical zones of old leaves in both species and, in *Clivia*, also in the basal zone, nuclei with dry mass contents corresponding to a level lower than 2C appeared. In the apical zone no nuclei with dry mass corresponding to the 4C level and in *Clivia* even to the 2C-4C level were found (Fig. 8a, b). The decrease in dry mass content in both species was greater in the apical zone.

DISCUSSION

During the development of a young leaf, mitotic divisions in the epidermis stop earlier than in the mesophyll, although the epidermis is thought to control growth and development of other leaf cells (Dale 1988). Outside the meristematic zone growth is accomplished only by elongation of cells (Cionini et al. 1983).

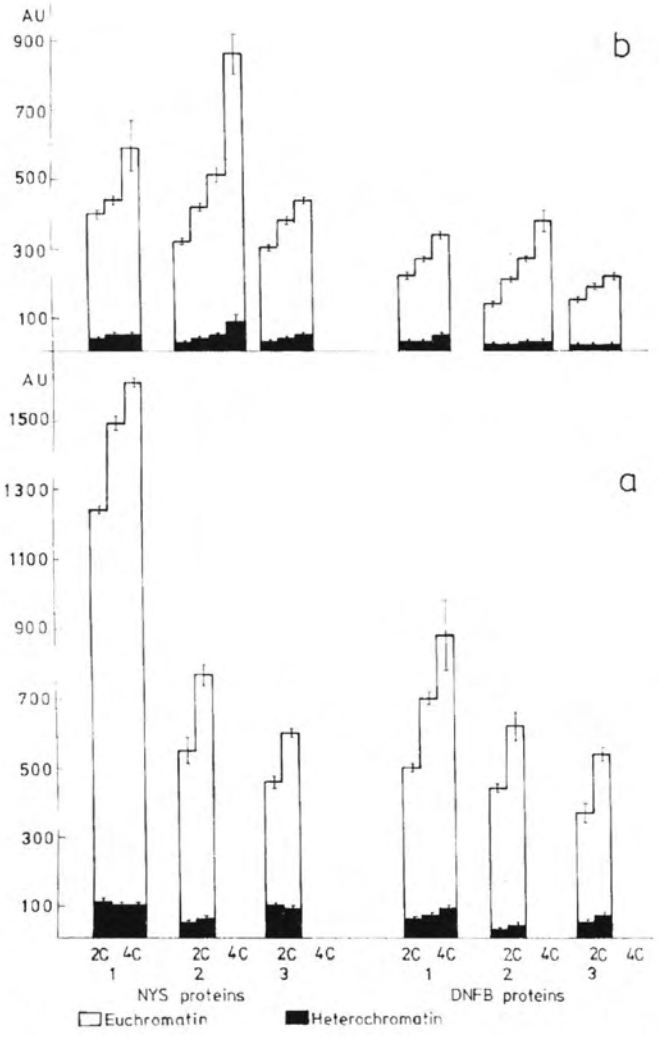

Fig. 6.
NYS and DNFB protein content.
1 – young leaf, basal zone;
2 – old leaf, basal zone;
3 – old leaf, apical zone.
a – *Clivia miniata*, b – *Rhoeo discolor*.
Ordinate – protein content (AU), secant – DNA content

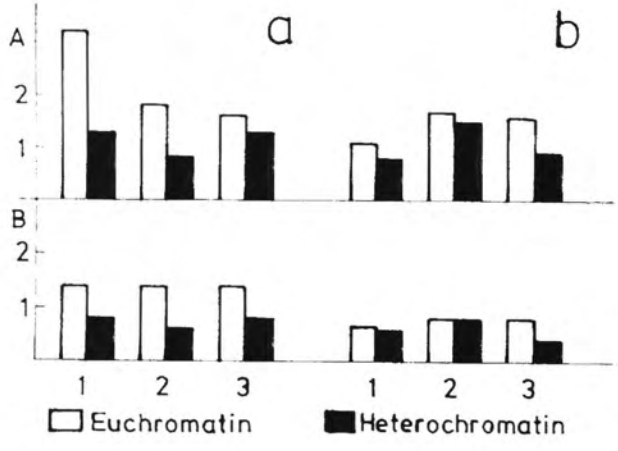

Fig. 7.
NYS proteins/DNA ratio (A) and DNFB proteins/DNA ratio (B).
1 – young leaf, basal zone; 2 – old leaf, basal zone;
3 – old leaf, apical zone.
a – *Clivia miniata*, b – *Rhoeo discolor*

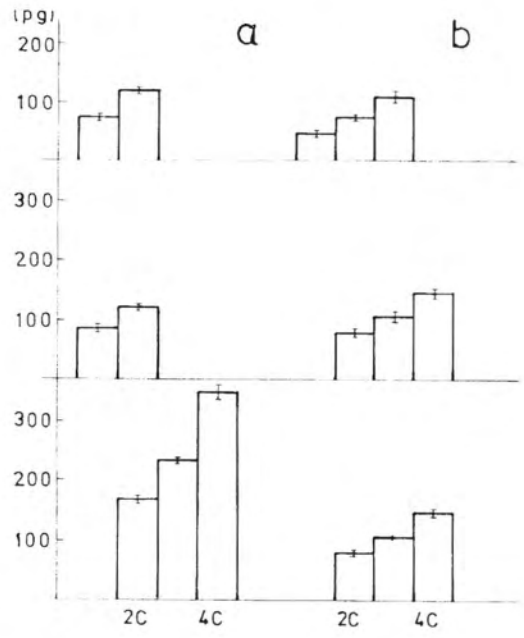

Fig. 8. Dry mass content.
1 – young leaf, basal zone;
2 – old leaf, basal zone;
3 – old leaf, apical zone.
a – *Clivia miniata*,
b – *Rhoeo discolor*.
Ordinate – dry mass content (pg), secant – DNA content (C values)

In both examined species, nuclear DNA content in the basal zone of young leaves was 2C, 2-4C and 4C DNA, characteristic for meristems. In the basal zone of old *Clivia miniata* leaves, the species with no endoreplication in roots (Olszewska and Osiecka 1982) or leaves (Olszewska et al. 1983), the level of DNA in epidermal nuclei was also 2C. A certain number of endoreplicating cells was present in the basal zone of very young leaves of *Triticum durum*, and although typical epidermal cells – interstomatal and interhairs – had 2C (Cionini et al. 1983), endoreplications reached different and high levels (up to 16C) DNA. Endomitotic polyploidy of nuclei may occur with both a high and low basic DNA content (Nagl et al. 1977). The maximum DNA level, 16C and even 32C, described in dicotyledonous herbaceous plants, occurred in both basal and apical leaf zones (Damsz and Łuchniak 1988). There were no significant differences in DNA content between the epidermis and mesophyll (Harris et al. 1982). In the basal zone of old *Rhoeo discolor* leaves, after the optimum time of hydrolysis for this species – 1h, nuclei with a 2-4C DNA content were found and a DNA content of 4C was reached in 13% of the nuclei. Noteworthy is also the fact that in *Rhoeo discolor*, endoreplication takes place in root parenchyma up to the 8C level (Olszewska et al. 1988).

A significant percentage of nuclei did not reach the 2C level in the apical and, sometimes, in the basal zones of old leaves in the tested species. A decreased DNA level in aging leaves of dicotyledonous plants was found in several species (Dhillon and Miksche 1981, Harris and Schaefer 1981, Harris et al. 1982, Olszewska et al. 1986). The greatest loss of DNA (34%) was found in the epidermis of *Nicotiana tabacum*; the decrease in the mesophyll DNA content occurred later than in the epidermis, suggesting that leaf aging may start in the latter (Harris et al. 1982). From among monocotyledonous annual species, DNA loss was found in *Zea mays* (Harris et al. 1984); the results of the present paper concerning two monoc-

otyledonous perennial species show that the same phenomenon is also observed in them. In the basal and apical zones of old leaves in these species, nuclei with 2C DNA contents dominate and the longer the duration of hydrolysis, the greater the number of these nuclei. Moreover, the decrease in DNA content in old leaves manifests itself in the presence of a considerable number of nuclei with a DNA content below 2C. Extinction in old leaves decreases significantly after each tested hydrolysis time. At the optimum hydrolysis time this decrease is highest and affects both euchromatin and chromocenters. DNA loss cannot be attributed to the presence of tannins (Greilhuber 1988) because the $K_2Cr_2O_7$ test was negative.

It is known that progressive condensation of chromatin is connected with decreased sensitivity to acid hydrolysis in the Feulgen procedure (Mello 1979, Olszewska et al. 1986 and ref.). In the present experiments, DNA content measured after each hydrolysis time decreased and prolongation of hydrolysis brought about a decrease in extinction values. The level of euchromatin condensation depends on interaction between DNA and basic proteins and the amount of strong bonds between DNA and proteins increases in the aging process (Maternickij 1989). In three *Rhoeo* species, the C-bands in metaphase chromosomes (Pettenati 1987) are identical with chromocenters in interphase nuclei in the basic genome. The ratio of euchromatin DNA to heterochromatin DNA in old leaves of *Rhoeo* decreased after 1h and 2h of hydrolysis, indicating that chromocenter DNA and euchromatin DNA are of different sensitivity. A significant increase in the ratio of euchromatin DNA to heterochromatin DNA after 4h of hydrolysis may be caused by the fact that part of the chromocenters decondensed and their DNA mistaken for euchromatin DNA. However, in *Zea mays*, the euchromatin/heterochromatin ratio does not change under the same conditions of hydrolysis in young and old leaves (Harris et al. 1984). The decrease in chromocenter DNA content was lower than that of euchromatin DNA in both tested species. It is known that euchromatin is less resistant to acid hydrolysis than heterochromatin (Mello 1979, Olszewska et al. 1986 and ref.). There was approximately 20% less heterochromatin in the nuclei of old *Arachis hypogea* leaf epidermis than in young leaves, after the same time of hydrolysis (Harris et al. 1982). The relatively low DNA content in the chromocenters of old leaves found in our material may be caused by considerable condensation of chromatin, as a result its of 25% greater density in old leaves (Dhillon and Miksche 1981).

The surface occupied by chromocenters in both tested species is greatest after the optimum time of hydrolysis. Further prolongation of hydrolysis decreases the size of chromocenters and the DNA content in them. The prolongation of hydrolysis causes a decrease in relative DNA content due to depolymerization; partial disappearance of chromocenters was also observed (Olszewska et al. 1986). The increase in the surface of chromocenters per nucleus in old leaves of both species after 1h and 2h hydrolysis is in agreement with the fact that aging of leaves is accompanied by an increase in the amount of condensed chromatin (Olszewska et al. 1986); an increase in the number of chromocenters was observed during the development of *Triatoma nymphs* (Mello 1979).

The cytophotometric measurement of proteins stained with NYS and DNFB in euchromatin and heterochromatin of old leaves shows, similarly to DNA content, a decrease in AU. A correlation between changes in protein and DNA contents was also observed during root differentiation (Olszewska et al. 1987). Staining with NYS, revealing mainly nonhistone proteins (Tas and James 1981), showed that in both species the greatest decrease appeared in the apical zone, that is, in that zone where the greatest decrease in dry mass occurred. A close relationship between the amount of protein-bound NYS and dry mass was also present in the nuclei of animal cells (Gaub 1981) and during the cell cycle (Olszewska et al. 1990). Harris et al. (1984) found a 14% decrease in the dry mass of nuclei from old maize leaves. The decrease in dry mass content, particularly visible in *Clivia*, described in this paper is largely due to the presence of as much as 86% of nuclei that did not reach the 2C level. The lower drop in NYS proteins in *Rhoeo* than *Clivia* may be due to the lack of endoreplication in *Clivia*. It is known that the synthesis of specific nonhistone nuclear proteins that take part in chromatin activation is lowered in old cells (Maternickij 1989).

In conclusion, the different times of hydrolysis used in the experiments described in this paper show that during aging, the decline in the DNA content is not due to progressing chromatin condensation but to DNA loss; it is accompanied by a decrease in dry mass and protein contents. This conclusion may be supported by the finding of increased DNase (pH 6.6) activity in old leaves (Kumar and Khan 1984). The symptoms of aging are observed mainly in the apical zone of an old leaf.

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ZMIANY W ZAWARTOŚCI DNA, SUCHEJ MASY I BIAŁEK W JĄDRACH KOMÓREK SKÓRKI LIŚCI PODCZAS STARZENIA SIĘ ROŚLIN JEDNOLIŚCIENNYCH WIELOLETNICH

STRESZCZENIE

U 2 gatunków roślin jednoliściennych wieloletnich – *Clivia miniata* i *Rhoeo discolor* – w jądrach skórki strefy bazalnej liści młodych oraz strefy bazalnej i apikalnej liści starych, określano cytofotometrycznie zawartość DNA przy zastosowaniu metody Feulgena (po 1h, 2h i 4h hydrolizy 4N HCl), zawartość białek barwiących się NYS i DNFB, oraz metodą interferometryczną zawartość suchej masy. Wykazano, że w obu strefach liści starych dominują jądra o zawartości 2C DNA oraz występuje znaczny procent jąder o zawartości DNA poniżej poziomu 2C. Stosunek DNA euchromatyny/heterochromatyny wskazuje na większy spadek zawartości euchromatyny podczas starzenia. Obniżeniu zawartości DNA towarzyszy spadek zawartości białek barwiących się NYS i DNFB oraz obniżenie zawartości suchej masy, skorelowane przede wszystkim ze spadkiem zawartości białek NYS. Wyniki uzyskane przy zastosowaniu zróżnicowanego czasu hydrolizy pozwalają przypuszczać, że podczas starzenia następuje rzeczywisty ubytek DNA.

SŁOWA KLUCZOWE: skórka liści, starzenie, jednoliścienne wieloletnie, zawartość DNA, sucha masa, zawartość białek, *Clivia*, *Rhoeo*