

DNA DAMAGE IN *CREPIS CAPILLARIS* CELLS IN RESPONSE TO IN VITRO CONDITIONS

JOLANTA KWASNIEWSKA*, WITOLD NAWROCKI, DOROTA SIWINSKA,
AND JOLANTA MALUSZYNSKA

Department of Plant Anatomy and Cytology, University of Silesia,
Jagiellonska 28, 40-032 Katowice, Poland

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We analyzed DNA damage, mitotic activity and polyploidization in *Crepis capillaris* callus cells during short- and long-term in vitro culture, and the influence of plant growth regulators on these processes. Changes in the concentration of growth regulators altered the stability of callus. The level of DNA damage was highly dependent on the growth regulator composition of the medium. Cytokinin at high concentrations damaged DNA in the absence of auxin. Short- and long-term callus differed in sensitivity to growth regulators. Mitotic activity changed when callus was transferred to medium with modified growth regulators. Callus cell nuclear DNA content increased with age and in response to plant growth regulators. Hormones played a role in the genetic changes in *C. capillaris* callus culture. We demonstrated the usefulness of *C. capillaris* callus culture as a model for analyzing the effect of culture conditions, including plant growth regulators, on genetic stability.

Key words: Callus, *Crepis capillaris*, DNA damage, plant growth regulators.

INTRODUCTION

Genetic instability is a major constraint in culture of plant cells in vitro. Genetic changes, including chromosomal instability in the callus phase, can produce somaclonal variation in regenerated plants (Phillips et al., 1994). These changes can also diminish cell viability and callus mass increase. The stability of cells in culture in vitro depends on culture conditions such as medium components, culture age and subculture intervals. Until now the cytogenetic stability of cells in culture has been assessed mostly by analyzing the level of polyploidization and the frequency of chromosome rearrangements.

Crepis capillaris is one of the few species characterized by diploidy during differentiation (non-polysomatic type); due to its simple karyotype ($2n=2x=6$) it has been widely investigated in vivo and in vitro. Callus culture of *C. capillaris* is relatively stable cytogenetically as compared with calli of other plant species (Vapper and Kallak, 1986). Changes in chromosome structure occur during the first year of culture, but within the original ploidy level (Sacristan, 1971; Maluszynska, unpubl. data).

In contrast, callus of *Arabidopsis thaliana*, a polysomatic species (Galbraith et al., 1991), shows a high level of polyploidization during early stages of callogenesis (Fras and Maluszynska, 2004; Fras et al., 2007). DNA fragmentation occurred in *A. thaliana* callus within the first few days of in vitro culture, and was attributed to redifferentiation and dedifferentiation of cells in the young callus rather than to senescence (Fras et al., 2007). Such changes may also indicate programmed cell death (PCD), which also occurs in response to conditions in vitro (Hao et al., 2004). There are no similar DNA-level studies on the genetic stability in *C. capillaris* callus cells, so it has not been determined whether they are subject to DNA damage in vitro.

In this study we aimed to discover any correlation between changes in mitotic activity, polyploidization and DNA damage levels in *C. capillaris* callus cells, on the one hand, and in vitro culture factors such as culture age and plant growth regulator concentrations on the other. We used the TUNEL test and comet assay to detect and analyze DNA damage levels. We used flow cytometry to examine the effects of callus age and growth regulators on ploidy level.

*e-mail: jolanta.kwasniewska@us.edu.pl

MATERIALS AND METHODS

MATERIAL

Leaves of *Crepis capillaris* (L.) Waller ($2n=2x=6$) were the explant source for callus induction. Leaves from plants growing in sterile conditions were dissected and placed on solid MS medium (Murashige and Skoog, 1962) with standard hormone supplementation: the auxin, 6.0 mg dm^{-3} naphthalene acetic acid (NAA, Sigma), and the cytokinin, 0.6 mg dm^{-3} kinetin (Sigma) (Sacristan and Wendt-Gallitelli, 1973). After the callus appeared it was dissected from the explants and subcultured every 4 weeks. Callus induction was repeated 7 months later. Two-month cultures (short-term callus) and nine-month cultures (long-term callus) were transferred to MS medium with different compositions of plant growth regulators (Tab. 1) and maintained for 3 months, with subculturing every 4 weeks. The cultures were kept under a 16 h photoperiod at 21°C .

We analyzed short-term callus transferred at two months to modified medium for one month (2+1), two months (2+2) or three months (2+3), and long-term callus transferred at nine months to modified medium for 3 months (9+3).

MITOTIC INDEX

The mitotic index was analyzed for all groups of callus growing on standard and modified media. To determine mitotic activity the callus was fixed in methanol and acetic acid (3:1 v/v) for 4 h at room temperature. Slides were prepared by Feulgen's squash technique. Five preparations from different pieces of callus were made for each experimental group, and the frequency of dividing cells was counted from 2000 cells on each slide.

FLOW CYTOMETRY

To analyze the nuclear DNA content of the callus cells we used a DAKO Galaxy flow cytometer equipped with an HBO-100 mercury lamp, and a Cystain UV precise P kit (Partec) containing isolation and staining buffers. Tissues growing on MS medium enriched with kinetin and NAA at different concentrations were chopped with a razor blade in isolation buffer. The samples were then filtered through a $50 \mu\text{m}$ nylon sieve (Partec Cell Trics), the nuclei were stained with DAPI, and the data were processed as histograms with FolMax software. Ploidy level was determined from 3 callus samples in each experimental group: callus aged 5 and 12 months growing on standard and modified medium for 3 months (2+3 and 9+3). Callus cells aged 5

TABLE 1. Concentrations of plant growth regulators added to MS medium

	NAA	kinetin
Standard medium	6 mg dm^{-3}	0.6 mg dm^{-3}
Modified media	6 mg dm^{-3}	1.2 mg dm^{-3}
	0	1.2 mg dm^{-3}

months growing for 3 months on modified medium without NAA and 1.2 mg dm^{-3} kinetin died, so were not analyzed.

TUNEL TEST

The TUNEL test was performed for all callus culture treatments. Cells of young leaves were used as control to exclude any pre-existing variation in callus (Lopez et al., 2010).

Samples of callus tissue and leaves were fixed with freshly prepared 4% paraformaldehyde (Fluka) in PBS for 1 h at RT, and the fixed material was then washed $3 \times 5 \text{ min}$ in PBS. Preparations of nuclei were made by squashing callus tissue in PBS buffer. After freezing at -70°C , preparations were stored for several days at 4°C .

Cells were permeabilized by incubating the preparations in 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate for 2 min at 4°C and rinsing with PBS. DNA fragment labeling was carried out with TUNEL reaction mixture (In situ Cell Death Detection Kit, Fluorescein, Roche). The positive control was a preparation of leaf nuclei treated with DNase solution (1U) for 30 min at 37°C in a humid chamber. The preparations were rinsed twice with PBS and DNA fragment labeling was done with the TUNEL reaction mixture. $50 \mu\text{l}$ of TUNEL reaction mixture (enzyme solution – terminal transferase, label solution, 1:9 v/v) was applied to the preparations; then they were incubated in a humid chamber in the dark for 1 h at 37°C . The negative control of the TUNEL reaction was a mixture without enzyme. The preparations were rinsed 3 times with PBS and stained with DAPI ($2 \mu\text{g dm}^{-6}$), air-dried and then mounted in Citifluor. The preparations were evaluated with a fluorescence microscope with filters for FITC and DAPI. Five preparations from different pieces of callus were used for each experimental group. Each preparation came from one callus sample. The frequency of labeled nuclei in the TUNEL test was established from 2000 cells of each preparation, and means were calculated for each group. The significance of differences between callus growing on control and modified medium was assessed with Student's t-test, with $P < 0.05$ taken as indicating significance.

COMET ASSAY

To optimize the parameters of the comet assay, 1- and 8-month-old callus (one subculture before transfer to modified medium) was treated with maleic acid hydrazide (MH; Sigma, CAS 123-3301) freshly dissolved in water to a final concentration of 1 and 4 mM MH. The control was leaf tissue. The plant material was treated with mutagen solutions for 2 h at 20°C and then rinsed 3 x 5 min in distilled water. Different pre-electrophoresis (PE) and electrophoresis (E) times were used to optimize the procedure: 8 min PE, 8 min E; 8 min PE, 15 min E; and 15 min PE, 15 min E.

To isolate nuclei, individual leaves and pieces of callus tissue were placed in a small Petri dish containing 200 µl cold 400 mM Tris-HCl buffer, pH 7.5, on ice. The callus was carefully cut with a razor blade to release nuclei into the buffer under yellow light. Each slide previously coated with 1% NMP agarose (Sigma) and dried was covered with a mixture of 55 µl nuclear suspension and 55 µl LMP agarose (1% prepared with phosphate-buffered saline, Sigma) at 40°C, and a coverslip was added. The preparation was then placed on ice for at least 5 min and the coverslip was removed. Then 110 µl LMP agarose (0.5%) was placed on the slide and the coverslip replaced, and removed again after 5 min on ice.

The slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and incubated for 15 min. Electrophoresis was performed at 15 V, 190 mA for 15 min. The gels were then neutralized by washing three times in 400 mM Tris-HCl, pH 7.5, and stained with DAPI (2 µg dm⁻⁶). After staining, the preparations were dipped in ice-cold distilled water and immediately analyzed.

The comet assay experiment was performed two times, using 5 preparations (each from one callus sample and one leaf) for each experimental group. For each preparation, 50 randomly chosen cells were analyzed under the fluorescence microscope with a filter for DAPI, using an image analysis system (Komet Version 5.5. Kinetic Imaging, Liverpool, UK). Tail DNA (TD, %) was used as the parameter of DNA damage, and the median TD values were calculated for each slide. Comet images were visually scored to optimize the comet assay parameters. Each of 100 comets per preparation was assigned to a category from A0 to A4 according to the degree of damage (A0 – undamaged nuclei, A4 – whole DNA in the tail). The overall score (A1+2A2+3A3+4A4; A1, A2 – number of comets in each category) was between 0 and 400 arbitrary units, where the maximum score of 400 indicates that all comets have maximal extension of DNA into the tail (Collins, 2004).

TABLE 2. Mitotic index of *Crepis capillaris* callus cells growing on standard and modified media. Data in percentages

Age of callus (months): on control medium + on modified medium	Standard medium	Modified media	
	6 mg dm ⁻³ NAA, 0.6 mg dm ⁻³ kinetin	6 mg dm ⁻³ NAA, 1.2 mg dm ⁻³ kinetin	0 mg dm ⁻³ NAA, 1.2 mg dm ⁻³ kinetin
2+1	2.9	1.8	0.5
2+2	2.6	2.2	0.05
2+3	3.0	4.0	-
9+3	1.8	2.5	3.9

Means ±SD for TD were calculated for each slide, and averaged from the replicate experiments of each experimental group. The significance of differences between groups was checked with Student's t-test, with P < 0.05 taken as indicating significance.

RESULTS

MITOTIC INDEX

The values of the mitotic index for calli from standard medium showed no significant difference for 3-, 4- and 5-month-old calli (~3%) (Tab. 2). The long-term culture (9+3) on standard medium showed lower mitotic activity: only 1.8% dividing cells.

The mitotic activity of callus transferred to modified medium changed in an age-dependent manner. Short-term callus grown for 2 months (2+1, 2+2) on medium with 6 mg dm⁻³ NAA and 1.2 mg dm⁻³ kinetin showed a decreased mitotic index. Unexpectedly, 2 month-old callus transferred to modified medium for 3 months showed an increased mitotic index. There was no such increase in mitotic activity in long-term callus transferred to this medium for 3 months, but the index was higher than for callus growing on standard medium.

Mitotic activity decreased in short-term callus transferred to modified medium with 0 mg dm⁻³ NAA and 1.2 mg dm⁻³ kinetin for 1 or 2 months. This callus was characterized by very slow growth. Callus transferred for 3 months to this medium stopped growing altogether. In contrast, the mitotic activity of long-term callus grown for 3 months on medium without NAA was twice that of callus on standard medium.

PLOIDY LEVEL

The histograms show that the ploidy level of callus growing on the standard medium increased with age (Fig. 1a,b). For callus from short-term culture grown

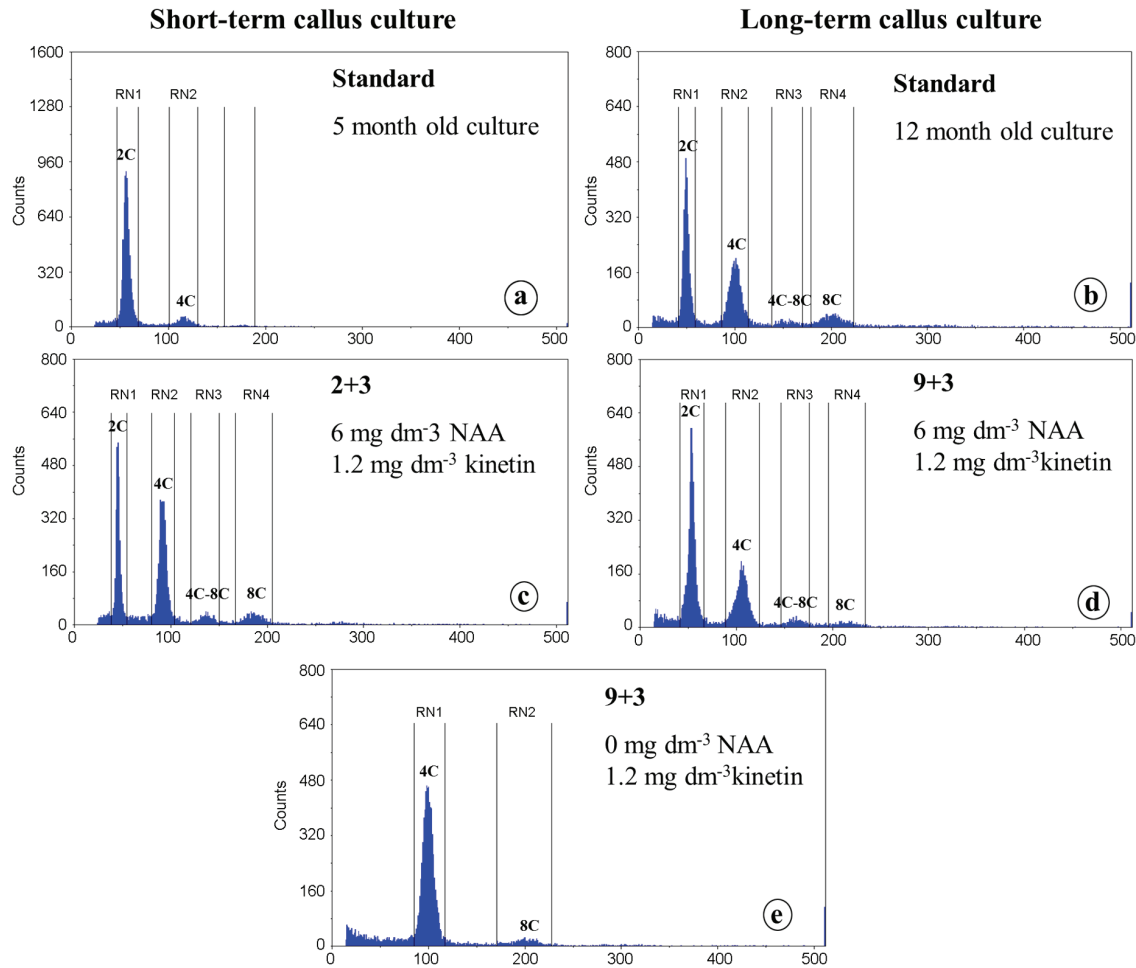


Fig. 1. Flow cytometric analysis of ploidy level of *Crepis capillaris* callus cells growing on standard MS medium (a, b), medium with double concentration of kinetin (c, d), medium with double concentration of kinetin and without NAA (e). Short-term callus culture (a), long-term callus culture (b, d, e).

on standard medium for 5 months, two peaks were distinguished – 2C and 4C, corresponding to G1 and G2 cells. In long-term culture (at 12 months) we confirmed 4C–8C and 8C peaks; more cells showed the 4C peak (Fig. 1b).

Changes in growth regulator concentration altered the stability of the callus. The number of peaks changed when it was transferred to the modified medium (Fig. 1c–e). In the callus grown for 2 months on standard medium and then for 3 months on medium with double the kinetin concentration and containing NAA, two small additional peaks appeared, and the number of cells representing the 4C peak increased. There are no results for 2-month-old callus transferred for 3 months to medium with double the kinetin concentration and without NAA, as this callus showed reduced growth and then died. Four peaks were observed for the 12-month-old callus growing on

standard medium as well as on modified medium with double the kinetin concentration (Fig. 1b,d). Only 2 peaks, 4C and 8C, were distinguished for callus aged 12 months growing for 3 months on medium with double the kinetin concentration and without NAA (Fig. 1e).

DNA DAMAGE

Tunel test

Our analysis of the effect of the applied growth regulators on DNA damage is based on examination of the frequencies of TUNEL-positive nuclei (Figs. 2e,f, 3). Positively labeled nuclei, indicating DNA fragmentation, were observed occasionally in leaf material (data not shown). In the nuclei of callus from standard medium, DNA fragmentation occurred at very low frequency (0.8–1.5%). Most of the nuclei (~90%)

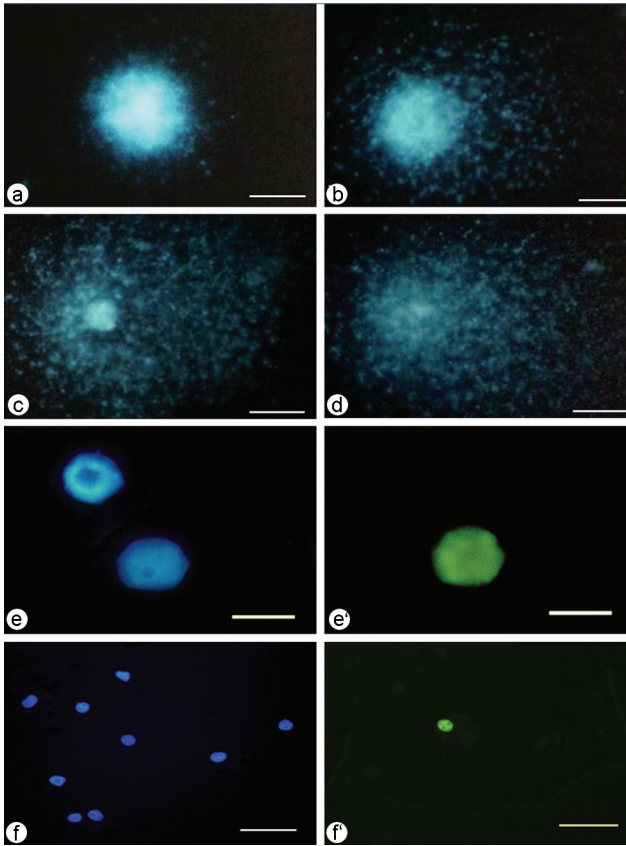


Fig. 2. *Crepis capillaris* callus cells. DNA damage expressed in comet assay (a–d) and TUNEL test (e, e' – DAPI, f, f' – TUNEL results). Bar = 10 µm.

showed green fluorescence when DNase solution was applied to the callus preparations (positive control).

The frequency of labeled nuclei of callus from standard medium did not change significantly during growth, and increasing the kinetin concentration in the presence of NAA did not induce DNA fragmentation either. The TUNEL test results showed that kinetin applied to the medium at high concentrations in the absence of NAA led to significant DNA damage. The response of long-term callus to this medium was weaker than that of short-term callus. The frequency of nuclei with DNA damage was highest in short-term callus growing for 3 months on medium with double the kinetin concentration and without NAA – 21.2%. When long-term callus was transferred to the same medium for 3 months (9+3) the frequency of labeled nuclei also increased (4%) but was lower than for short-term callus.

Comet assay

To optimize the parameters of the comet assay, 1- and 8-month-old callus (one subculture before

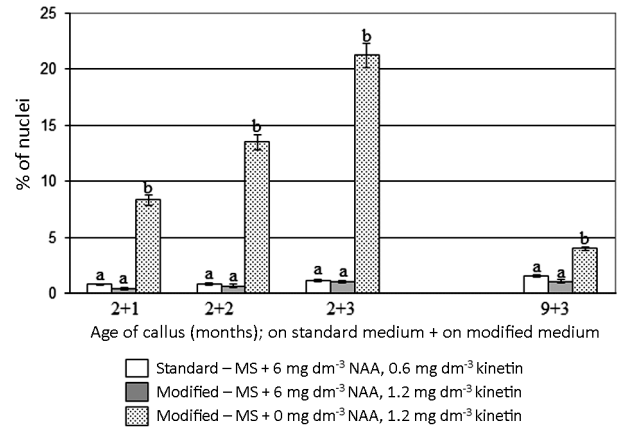


Fig. 3. Frequency of TUNEL-positive nuclei in *Crepis capillaris* callus cells growing on standard and modified MS medium. Values with different letters within the same culture differ significantly by Student's t-test at $P < 0.05$.

transferring callus to modified medium) was treated with maleic acid hydrazide (MH). MH is a clastogen, demonstrated to induce DNA fragmentation (Kwasniewska et al., 2012). The electrophoresis conditions chosen for further experiments, 15 min PE and 15 min E, proved optimal as they showed a low level of DNA damage in callus cells from standard medium and a dose-dependent response after treatment with maleic acid hydrazide (Fig. 4).

LEVEL OF DNA DAMAGE IN CALLUS IN RESPONSE TO IN VITRO CONDITIONS

We used the comet assay to detect and analyze the level of DNA fragmentation in *C. capillaris* callus cells during in vitro culture under standard and modified hormone supplementation. Examples of nuclei with different levels of DNA damage in the comet assay are presented in Figure 2a–d.

The level of DNA fragmentation in cells growing on standard medium increased with age in subsequent passages. The level of DNA damage was low in 3-month-old callus, increased in 4- and 5-month-old calli, and was higher in 12-month-old than in 5-month-old culture (Fig. 5). The comet assay showed that DNA damage in callus cells changed during one subculture. The level was generally higher 1 week after subculturing than 3 weeks after subculturing; changes in the level within one subculture depended on the age of the cells. The differences in the level of DNA damage within one subculture were higher in short-term than in long-term cultures (Fig. 5).

Changes in the growth regulator composition of the medium affected the level of DNA damage in callus cells (Fig. 6). The level was highest in cells grow-

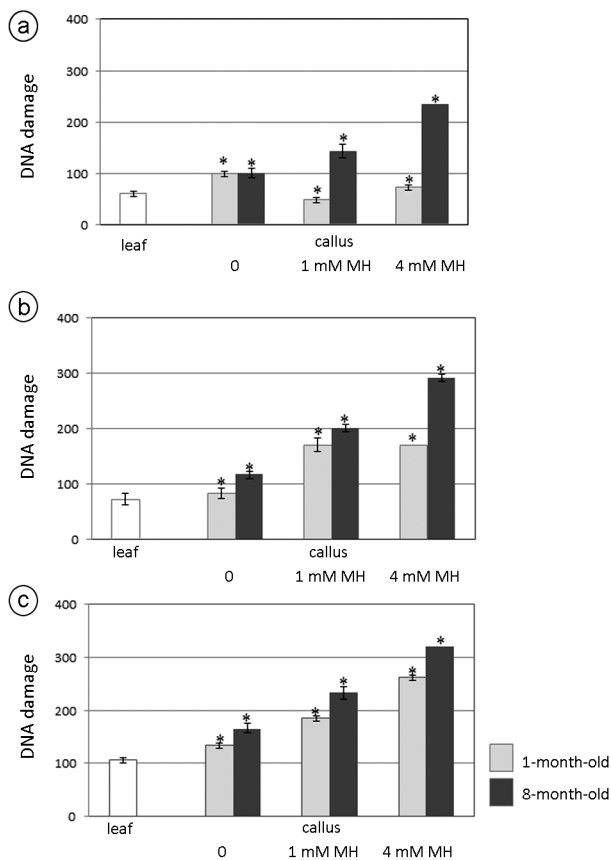


Fig. 4. Optimization of comet assay parameters. (a) 8 min unwinding, 8 min electrophoresis, (b) 8 min unwinding, 15 min electrophoresis, (c) 15 min unwinding, 15 min electrophoresis. DNA damage expressed in arbitrary units. *indicates groups within the same callus age class differing significantly by Student's t-test at $P < 0.05$.

ing on medium supplemented with double the kinetin concentration and without NAA. The reaction of *Crepis* calli to medium with modified growth regulator concentrations depended on the age of the cells. The level of DNA fragmentation increased significantly only in short-term callus. For example, TD for short-term callus nuclei from standard medium in the 5th month was 4.4%; TD for short-term callus transferred for 3 months to medium with 1.2 mg dm⁻³ NAA and without kinetin was about three times higher at 13.7%. The level of DNA damage in long-term callus transferred to modified medium did not change significantly.

DISCUSSION

Here we showed that DNA damage in *C. capillaris* callus growing in standard conditions depends

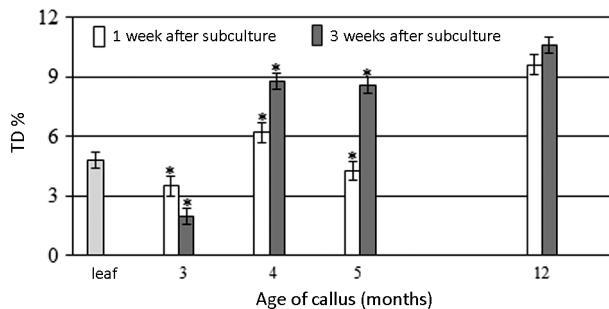


Fig. 5. DNA damage level in *Crepis capillaris* callus cells growing on standard MS medium (with 6 mg dm⁻³ NAA and 0.6 mg dm⁻³ kinetin), analyzed 1 and 3 weeks after subculturing. DNA damage level expressed as % of DNA in tail (TD). *indicates groups within the same callus age class differing significantly by Student's t-test at $P < 0.05$.

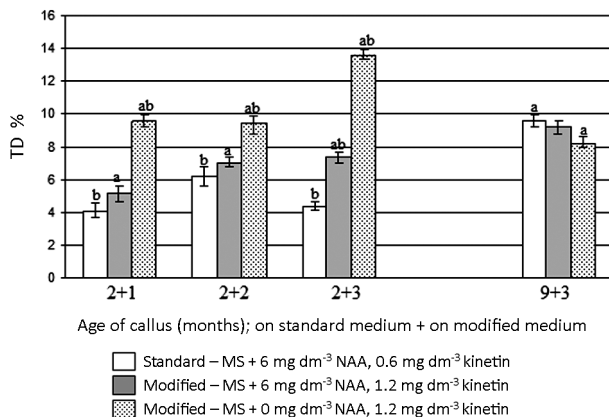


Fig. 6. DNA damage level (% TD) in *Crepis capillaris* callus cells growing on standard and modified MS medium. Values with the same letter within the same callus age class differ significantly by Student's t-test at $P < 0.05$.

strongly on the age of the culture, increasing with successive passages of culture in vitro. The lack of significance of increases in DNA damage in the early stage of callus culture (3rd month) is evidence that *C. capillaris* callus tissue is stable in its response to conditions in vitro in early culture. Our data are interesting in light of the reported chromosomal stability of *C. capillaris* callus in long-term culture (Sacristan, 1971). Structural chromosome aberrations have been reported in early callus culture (Maluszynska, 1990). Our work, which shows a lack of DNA damage together with changes at the cytological level, clearly indicates the operation of an efficient DNA repair process (Pfeiffer et al., 2004). The cells are not eliminated from the cell cycle, and this is why chromosome aberrations are detected (Marchetti et al., 2002). Several different events,

including DNA damage, have been identified in newly formed *A. thaliana* callus (Fras et al., 2007), but other studies have shown changes in chromosome structure and number during long-term in vitro culture (Joachimiak et al., 2003). For an explanation one may look to the fact that after explants are placed in the in vitro environment the cells adapt to the new conditions, resulting in genetic alterations (Elena and Lenski, 2003).

The level of DNA damage in callus cells growing on standard medium, analyzed in the comet assay, showed that it changed within one subculture, especially in short-term culture. The changes in the level of DNA damage observed during one subculture may be correlated with exhaustion of nutrients, as well as the accumulation of metabolic products in the medium; in this context we note that the level of DNA damage decreased after the tissue was transferred to new medium. Our results confirm that callus, which represents a heterogeneous culture, is, like cell-suspension culture, a good system for studying the mechanisms of DNA damage.

Our results connect hormones to DNA damage in *C. capillaris* callus culture. The level of DNA damage strongly depended on the growth regulator composition of the medium. Miller et al. (1955) showed that cytokinin, a phytohormone that controls plant growth and development, induces cell division. Another early study found that both auxin and cytokinin are required in culture media for cell division and callus formation to occur (Skoog and Miller, 1957). Recent studies stress how the interactions between different classes of hormones influence plant growth and development (Ross and O'Neill, 2001). Auxins and cytokinins act synergistically to regulate cell division (John et al., 1993; Coenen et al., 1997; Hartig and Beck, 2006). Our results confirmed that in the absence of auxins the mitotic activity of callus cells is reduced, and calli do not grow even if cytokinins are present. Cytokinins in interaction with auxin are required for cell division. Interestingly, changes in mitotic activity in response to modified medium without auxin were identified only in short-term callus, indicating its higher sensitivity than long-term callus. Changes in mitotic activity were also observed when callus was transferred to medium with auxin and a double cytokinin concentration; this involves the complex interaction of plant growth regulators with cell cycle gene expression (Richard et al., 2002). Auxin was found to arrest cells in G2, while cytokinin exerted that effect in G1 and G2 (Agostino and Kieber, 1999). Cells transferred to medium containing cytokinin and lacking auxin were arrested in G1 and G2 (Boer and Murray, 2000). The low mitotic activity of the callus cells transferred to modified medium might be explained similarly. Cells may skip the M phase, and the resulting endocycle leads to an

increase in the degree of polyploidy (Joubes and Chevalier, 2000).

To analyze the changes in ploidy we used flow cytometry. This method is particularly valuable for analysis of ploidy level in these callus cells, with their low mitotic activity. The presence of the additional peaks (4C, 8C) in callus from medium with increased kinetin concentration may be the result of endoreduplication. The 4C peak may represent the G1 phase of tetraploid cells, or the G2 phase of cycling cells. The latter suggestion finds support in the relatively high mitotic activity of the long-term callus cells. Short-term callus culture transferred to modified medium without auxin showed low mitotic activity; its first additional peak rather represents the first endoreduplication.

High cytokinin concentrations block cell proliferation and induce cell death (PCD) characterized by DNA fragmentation (Carimi et al., 2003). Other hormones are known to protect cells against cytokinin-induced death (Carimi et al., 2003). The comet assay is the best method for evaluating the level of DNA damage in individual cells (Lesniewska et al., 2000), although the TUNEL test has proved useful in studying callogenesis in *A. thaliana* (Fras et al., 2007) and citrus (Hao et al., 2004).

This is the first report of application of the comet assay for *C. capillaris* callus cells. Our results make it clear that the presence of high cytokinin concentrations in medium without auxin leads to DNA fragmentation. We showed that long-term callus apparently is less sensitive than short-term callus to plant growth regulators. This might be explained by a phenomenon whereby callus cultures lose their requirement for cytokinin in the course of growth (Pischke et al., 2006). The habituation process, defined as loss of the requirement for growth hormones by cultured cells, might also be responsible (Meins, 1989).

The DNA-damaging action of cytokinin in plant cells has been documented before, but in callus cells it is less well explored. More detailed knowledge of DNA damage in callus can help answer questions about aspects of somaclonal variation, including the role of growth regulators. Recently it was shown that cytokinins can trigger apoptosis (Mlejnek et al., 2002) characterized by DNA fragmentation in cell cultures. Earlier it was reported that two other hormones, 2,4-D and abscisic acid, protect cells against cytokinin-induced death characterized by DNA fragmentation (Carimi et al., 2003); in our studies the auxin (NAA) probably played a protective role. We used two methods, the TUNEL test and comet assay, to detect DNA fragmentation in callus cells in response to the presence of cytokinin at high concentration in medium without auxin. TUNEL gave the frequencies of callus cells with DNA damage, and the comet assay indicated the level of DNA damage

expressed by tail DNA. The TUNEL test showed that growth on modified medium with high kinetin concentrations and with NAA did not change the frequency of cells with DNA damage. The comet assay, on the other hand, showed increased DNA damage. The apparently divergent results indicate that the level of DNA damage, rather than the frequency of callus cells with DNA breaks, increases during culture on medium with a modified plant hormone composition. Together with the low mitotic index values, these data indicate that cells lose their capacity for cell division at high levels of DNA damage.

In this study we analyzed DNA damage in callus cells during in vitro culture and assessed the effect of plant growth regulators on this process. We found correlations between changes in mitotic activity, polyploidization, and the level of DNA damage in *C. capillaris* callus cells in response to plant growth regulators. We demonstrated the DNA-damaging effect of high cytokinin concentrations in the absence of auxin, and the usefulness of *C. capillaris* callus culture as a model for studying the effects of in vitro culture conditions, including plant growth regulators, on genetic stability.

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