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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Effects of exogenous glutathione on suspension callus cells of spruce [*Picea abies* (L.) Karst.]

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Callus cells of *Picea abies* (L.) Karst. were exposed to different concentrations (50, 100, 500, 1000 μM) of reduced glutathione (GSH) for 48 hours. These physiologically relevant concentrations of glutathione caused changes in the investigated tissue depending on the concentration applied. Feeding of glutathione increased the cellular concentrations of thiols, decreased the rate of cell division, induced mitotic abnormalities, generated increased amounts of micronuclei and affected the cell ultrastructure. The glutathione system in the callus culture cells was measured by a quantitative image analysis method, using histochemical staining by monochlorobimane. This measurement showed an increase of thiols at the cellular level after GSH treatment. Whereas no distinct structural modifications were found in cells treated with 50 and 100 μM, the treatment with 500 and 1000 μM GSH had multiple effects on the investigated tissue in comparison to control cells. Damage observed in the electron microscope involved separation of the plasma membrane from the cell wall, swollen plastids and mitochondria, and heavily granulated chromatin in the nuclei. The investigation of the chromosomal aberrations showed an increased amount of chromosomal defects in the GSH treated cells. The chromosomal aberration types observed most frequently were defects in the form of sticky chromosomes and vagrant chromosomes indicating severe damages in the genetic material.

Keywords: Glutathione, callus, *Picea abies*, monochlorobimane, ultrastructure, chromosomal aberrations

Introduction

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is found in most prokaryote and virtually all eukaryote cells, where it often represents the major pool of non-protein reduced sulphur (KUNERT and FOYER 1993). Numerous functions have been attributed to glutathione (GSH). It plays an important role in protecting plants against xenobiotics, heavy metals, and oxidative stress (NOCTOR et al. 1998). It is a major compound in sulphur metabolism acting as the main carrier of reduced sulfur and as a regulator of the interorgan

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sulphur allocation (cf. TAULAVUORI et al. 1999, HERSCHBACH and RENNENBERG 1997, 2001). In addition to its function as a substrate in phytochelatine synthesis and in reactions catalysed by GSH S-transferases, there is growing evidence that GSH also influences the control of gene expression and the cell cycle (MAY et al. 1998, NOCTOR et al. 1998).

The glutathione contents in plants can be affected by different environmental conditions such as the sulphate supply (DE KOK and KUIPER 1986, HERSCHBACH et al. 1995), light (BIELAWSKI and JOY 1986, NOCTOR et al. 1997), oxidative stress (MAY et al. 1998), exposure to heavy metals (CHEN and GOLDSBOROUGH 1994) or atmospheric pollution (LUWE 1996). The biochemical mechanisms through which external factors modulate the GSH contents have not yet been characterised. Even the regulatory processes that enable a concerted action of GSH in different functions within and beyond subcellular compartments are unknown. In many plants, one of the major difficulties in studying the control of glutathione synthesis is the low activity of the extracted enzymes. In addition, a precise determination of the GSH contents is difficult due to the fact that the GSH concentration in organelles can be influenced by a possible exchange of GSH between the different cell compartments during subcellular fractionation (KLAPHECK et al. 1987). Recently, histochemical methods involving the conjugation of the specific fluorescence dye bimane with thiol groups were used to demonstrate GSH concentrations at the tissue or even at the sub-cellular level (SÁNCHEZ-FERNÁNDEZ et al. 1997, MÜLLER et al. 1999a, MEYER and FRICKER 2000).

Over-expression of the enzymes of glutathione metabolism has opened the door to novel possibilities for industrial exploitation of GSH as for instance a flavour precursor in food (NOCTOR et al. 1998). Furthermore, glutathione is offered as a health product by different pharmaceutical companies.

The effects of exogenously applied glutathione on plant tissues are not clear. SÁNCHEZ-FERNÁNDEZ et al. (1997) showed a positive effect of 0.01 – 0.1 mM GSH on the meristematic activities of *in vivo* cultivated *Arabidopsis* root tissue. In contrast, data from experiments with young spruce trees suggested that 0.1 – 0.5 mM GSH caused severe damage to certain cell structures (MÜLLER et al. 2000, ZELLNIG et al. 2000).

Hence, the aim of this study was the investigation of the effect of GSH on suspension culture callus cells. This plant material grows in stable conditions and the externally applied GSH can be brought in direct contact to the cells. With this experimental set-up the effect of different GSH-concentrations in the liquid medium should be demonstrated more precisely because the investigated small cell clusters are equally surrounded by the GSH-medium and gradients that might occur in whole organs are to a very large extent avoided.

Material and methods

Cultivation of callus and GSH treatment

Fresh seeds of *Picea abies* (L.) KARST. were sterilized according to the method described by MESSNER and BERNDT (1990) and germinated on Perlite under sterile conditions. After 3 weeks under climate chamber conditions the cotyledons of the seeds were cut off, sterilized once more and transferred to petri dishes filled with the medium described below. Approximately one month later the callus cultures were removed from the cotyledon explants and sub cultured every three weeks.

The medium (1l) contained vitamins, macro-, and micronutrients as described by MURASHIGE and SKOOG (1962) (ready-to-use-mixture obtained from Duchefa, Nr. M0222), 30 g saccharose, and 1.1 g Agar Agar as gelling agent. The pH was adjusted to 6.5, the medium was then autoclaved at 125 °C for 20 min, cooled to approximately 45 °C, complemented with 3 mg/l naphthaleneacetic acid and 1 mg/l 6-benzylaminopurine, and put in sterilized petri dishes (about 40 ml per dish).

Sub-cultivation was carried out every one to two weeks under sterile conditions in a laminar flow box, brown tissue removed with a scalpel, each callus divided in 3 to 4 pieces (approximately 5 × 5 mm) and put on a liquid fresh medium (5 pieces per dish). The petri dishes were then sealed with stripes of parafilm. Incubation temperature was 20 °C, with a 12 h per day photo period.

Pieces of the callus were transferred to Erlenmeyer flasks, which were filled with liquid sterile MS medium. The cultures were grown on a rotary shaker. On day 10 the suspension cultures were treated with 50, 100, 500 and 1000 μM GSH in the culture medium for 48 hours with an exchange of the GSH-solutions every 12 hours. One culture remained untreated as a control. All the variants of the experiment remained unshaken to prevent oxidation of GSH.

Fluorescence microscopy

Control samples and 1000 μM GSH treated samples were stained with a 50 μM solution of monochlorobimane (BmCl) for 5 min at room temperature (modified according to MÜLLER et al. 1999b). After rinsing in 0.1 M phosphate buffer for 30 seconds to remove excess staining solution the samples were observed under the fluorescence microscope (Zeiss, Axioskop, 50 W mercury arc lamp), equipped with a 3-chip-color video camera (Sony DXC 930 P with Sony-control-system) and a frame grabber (ITI MFG-3M-V, Imaging Technology Inc., with variable scan module AM-VS-VP and colour recording module AM-CLR-VP). The image analysis software was Optimas 6.5.1. (BioScan Corp.). For BmCl fluorescence, cells were excited at 365 nm and the fluorescence was imaged through a 395 nm dichroic mirror and a 420 nm long pass filter. Luminescence profiles of control and GSH treated cells were made and the values were measured on a 0–255 digital scale after background subtraction. Differences in GSH content between control cells and GSH treated cells were shown by comparing these profiles. No software filters were used for these measurements.

Electron microscopy

Segments of the callus cultures were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer containing 0.1 M sucrose (pH 6.5) for 2h. After rinsing in the buffer, segments were post fixed in 1% (v/v) osmium tetroxide in 0.1 M phosphate buffer (pH 6.5) for 90 min. Subsequently, samples were dehydrated in a graded series of ethanol and propylene oxide and embedded in Agar 100 epoxy resin.

The tissue of five selected segments per sample was used for ultrathin sectioning. 80 nm ultrathin sections were post-stained with lead citrate and uranyl acetate and viewed with a Philips CM 10 electron microscope.

Cytogenetic studies

After exposure, the callus cells were fixed in ethanol : glacial acetic acid (3:1, v/v) for at least 24 hours. After fixation, the cells were hydrolysed in 3M HCl for 3 min at 63 °C, stained in freshly prepared Schiff's reagent and squashed in a few drops of 45% acetic acid (modified according to MÜLLER et al. 1991). Eight replicate samples were taken.

The cells in metaphase and anaphase were grouped according to FISKESJÖ (1994) for the *Allium*-test: metaphase, early anaphase, anaphase, fragments, vagrant chromosomes, sticky chromosomes and bridges, the last two were filed under stickiness. About 100 meta- and anaphases (from cells with intact cell walls) per replicate were examined for chromosomal aberrations. The percentages of abnormalities in total meta- and anaphases were calculated (aberration rate).

For the determination of mitotic index and number of interphase cells with micronuclei, 1000 cells per replicate sample were analysed. The percentage of micronuclei containing interphases in total interphases were calculated as the micronuclei rate. Mitotic index was expressed as the number of mitotic stages in total meristem cells (interphases and mitotic stages).

Statistics

Statistical analyses were completed using the Statistica (Stat-Soft, USA, 1994) software package. Effects of glutathione treatment on the cytological parameters were evaluated with the help of the Kruskal-Wallis test, followed by post-hoc comparisons according to Conover (BORTZ et al. 1990). $P < 0.05$ was regarded significant.

Results

Ultrastructure

In the cells of control callus cultures, large nuclei with a diameter of 15–20 μm and a high number of plastids and mitochondria were the most frequent cell components (Fig. 1). The mitochondria occurred in a large number, were longish and spherical shaped with a size of 0.4–2.5 μm on the section and in their dense matrix well developed cristae, as well as distinct regions of DNA, could be observed. Plastids had an average size of about 2.5–5 μm and showed a dense stroma, single internal membranes, some plastoglobuli, ribosomes, and occasional starch grains. In addition, a pronounced endoplasmatic reticulum (ER) system appeared within the cytoplasm and around the nucleus. Some small vacuoles containing electron-opaque material were observed throughout the control cells.

The cells treated with 50 and 100 μM showed ultrastructural consistency similar to that of the control cells.

Treatment with 500 and 1000 μM GSH for 48 hours induced severe changes in the callus cells. The cells showed signs of plasmolysis with separations of the plasma membrane from the cell wall (Fig. 2). Small vesicles were found in large numbers close to the plasma membrane (Fig. 4). Their size increased with the distance from the plasma membrane, their occurrence coming closer to the cell centre. Within the cytoplasm of the highly vacuolated cells large nuclei with heavily granulated chromatin occurred. Once also a small nucleus separated by dense cytoplasm from the main nucleus was visible (Fig. 3). The small

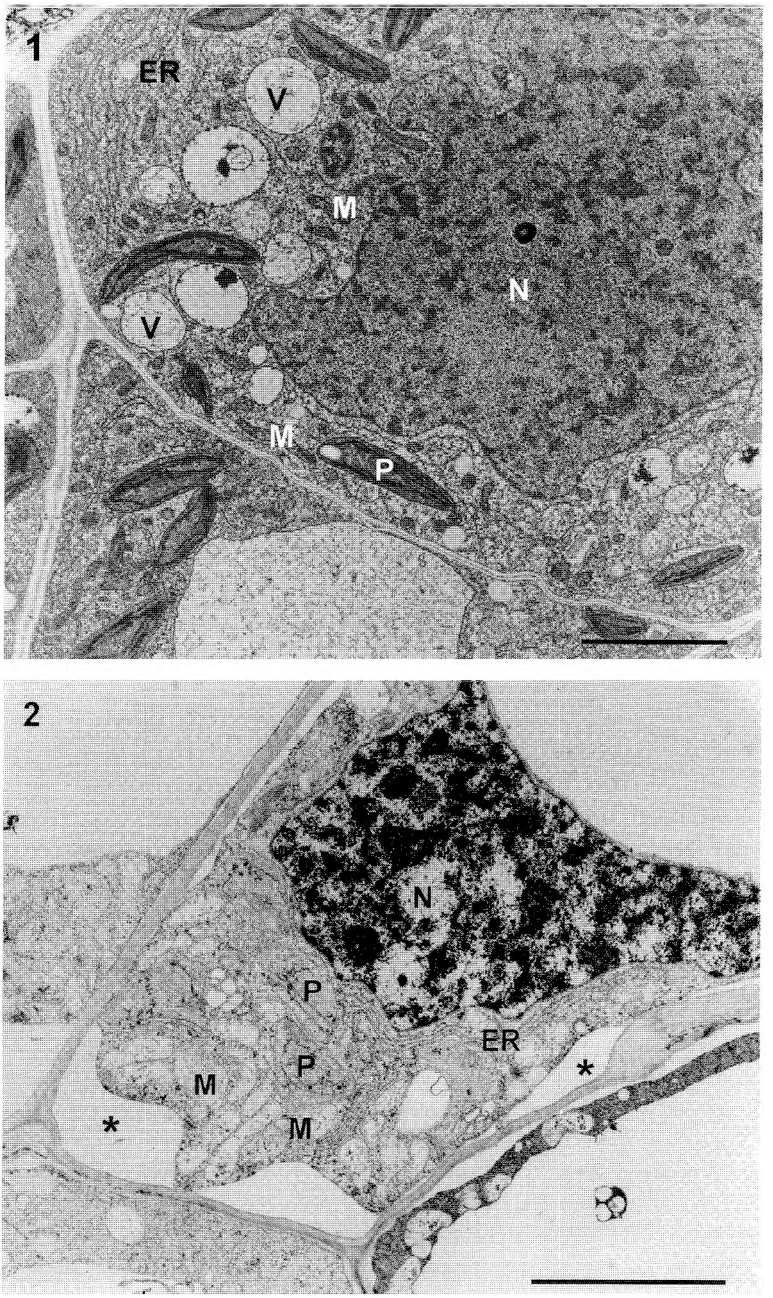

Fig. 1. Control callus cell with plastids (P), mitochondria (M), a nucleus (N), small vacuoles (V) and rough endoplasmic reticulum (ER). Bar = 5 μm.

Fig. 2. Callus cell treated with 1000 μM GSH for 48 h: The cell shows separations of the plasma membrane from the cell wall (*), swollen plastids with a loosened stroma (P), mitochondria with a loosened matrix (M), a nucleus with granulated chromatin (N) and the rough endoplasmic reticulum (ER). Bar = 5 μm.

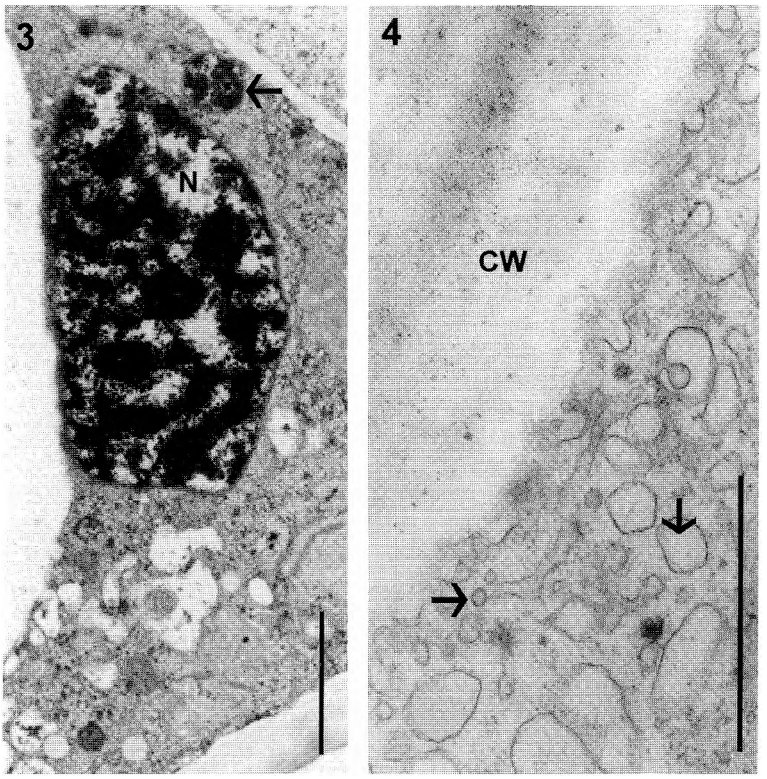

Fig. 3. Callus cell treated with 1000 μM GSH for 48 h: Besides the nucleus (N) a micronucleus (arrow) is visible, both structures contain heavily condensed heterochromatin. Bar = 2 μm.

Fig. 4. Callus cell treated with 500 μM GSH for 48 h: Vesicles of different diameter (arrows) can be found near the cell wall (CW). Bar = 2 μm.

nucleus had a size of about 1.2 μm and probably represents a so-called micronucleus. Often the irregularly shaped mitochondria and plastids were swollen and contained a loosened matrix or stroma which made those organelles sometimes difficult to identify. Interestingly, the structure of the internal membranes in the plastids seemed to be unaffected. The pronounced ER system remained unchanged and could be found within the cytoplasm and around the nuclei.

Glutathione quantification by fluorescence microscopy

In general the treatment with BmCl produced a strong blue fluorescence in the cytoplasm and in the nucleoplasm of the callus cells upon UV excitation and demonstrated the GSH content at the cellular level in vivo (Fig. 5a). The cell walls themselves were not fluorescent. Cells that were treated with GSH for 48 hours showed an increased fluorescence intensity all over the cells compared to the control cells (Fig. 5c). This increase of fluorescence yield in GSH treated cells is represented in the luminescence profiles (Fig. 5b, d) and indicated an increased concentration of low molecular weight thiols.

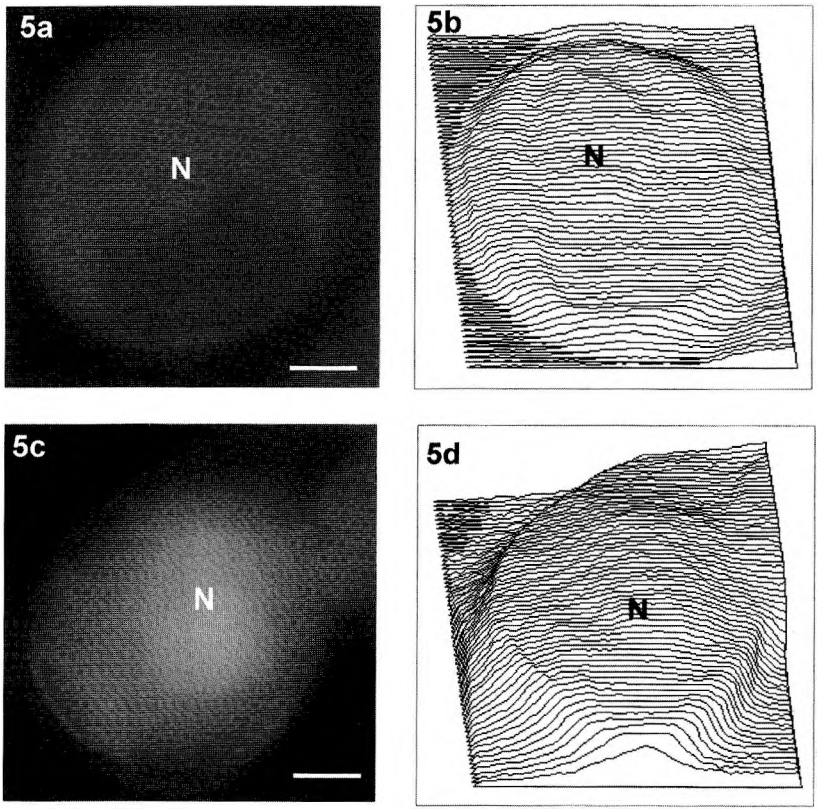

Fig. 5a-d. Micrographs of callus cells after incubation with 50 μM BmCl for 5 min showing fluorescence in the cytoplasm and in the nucleus (N). a -Control callus cell; b – luminescence profile of a. The luminescence intensity [arbitrary units] is shown at the vertical axis over the horizontal micrograph shown in a; c -Callus cell treated with 1000 μM GSH for 48 hours showing increased fluorescence in cytoplasm and in the nucleus (N); d -luminescence profile of c. The luminescence intensity [arbitrary units] is shown at the vertical axis over the horizontal micrograph shown in c. Bar = 10 μm.

Cytogenetic studies

The mitotic index was depressed upon all glutathione treatments (Fig. 6).

An increase in chromosomal aberration rates was induced by exposure to the different glutathione concentrations (Fig. 7). Controls had the lowest aberration rates, amounting to about 5% and an increase was induced by the exposure to 100 and 1000 μM GSH. The most common mitotic aberrations in all variants of the experiment were stickiness in the form of sticky chromosomes in the metaphase and anaphase bridges and also vagrant chromosomes. Other defects such as fragments in the meta- and anaphase could only be observed in response to the highest GSH concentration. The data of the 50 and 500 μM GSH concentrations were not determined because of the low mitotic index (Fig. 6).

The number of micronuclei increased significantly upon all glutathione treatments, up to 16% in the 100 and 1000 μM GSH concentrations (Fig. 8).

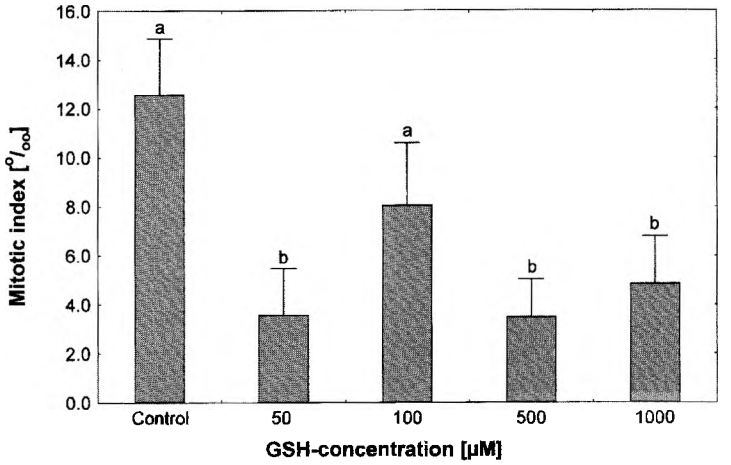

Fig. 6. Mitotic indices of callus cells exposed to different GSH concentrations for 48 hours. Significant differences among the variants of the experiment are indicated by different lowercase letters. Differences are calculated by Kruskal-Wallis variance followed by Conover's cross comparison. Data are medians and 80 percentile ranges.

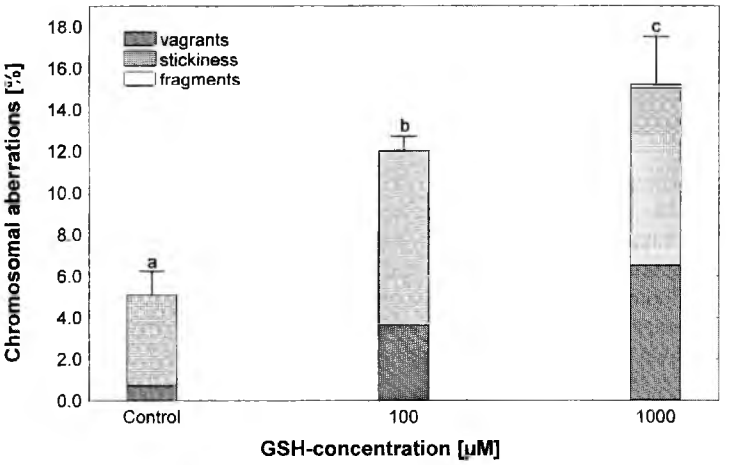

Fig. 7. Chromosomal aberrations in callus cells exposed to different GSH concentrations for 48 hours. Significant differences among the variants of the experiment are indicated by different lowercase letters. Differences are calculated by Kruskal-Wallis variance followed by Conover's cross comparison. Data are medians and 80 percentile ranges.

Discussion

The exogenously applied glutathione concentrations were in the magnitude of physiologically occurring millimolar tissue concentrations in plants (JAMAÏ et al. 1996, NOCTOR and FOYER 1998). The observed effects of external GSH were an increase of cellular low

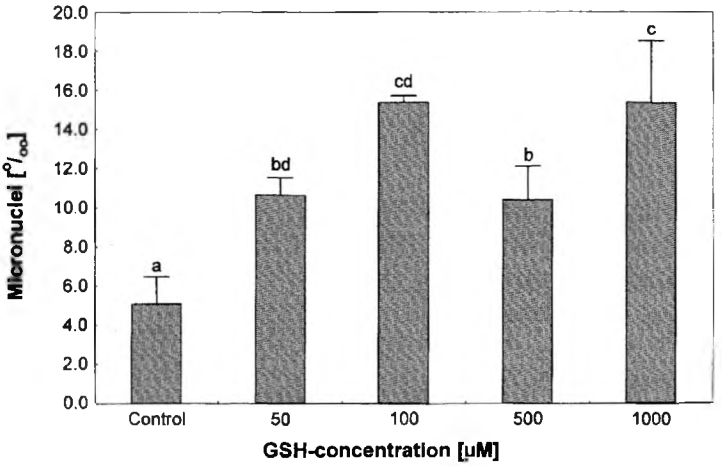

Fig. 8. Micronuclei in callus cells exposed to different GSH concentrations for 48 hours. Significant differences among the variants of the experiment are indicated by different lowercase letters. Differences are calculated by Kruskal-Wallis variance followed by Conover's cross comparison. Data are medians and 80 percentile ranges.

molecular thiols, severe changes of the cell ultrastructure, an affected cell division, and induced mitotic abnormalities in the form of chromosomal aberrations.

Cells that were treated with GSH for 48 hours showed an increased fluorescence intensity in the cytoplasm and nucleoplasm compared to the control cells, demonstrating an enhanced level of glutathione in the investigated cells. The problem of non-specific staining of protein-SH groups was overcome by the short incubation time of 5 min, since the –SH groups of small molecules, such as GSH and cysteine, react with the SH reagents at a fast rate, whereas the spontaneous reaction with other cellular thiols is very slow (e.g. COOK et al. 1991). BmCl does react rapidly with cysteine, but the cellular cysteine levels are generally much lower than GSH concentrations (RENNENBERG 1997), as has been also demonstrated in GSH spruce seedling feeding experiments by biochemical measurements of thiols in the root tissues (ZELLNIG et al. 2000).

The mitotic index, an indicator of the mitotic activity, demonstrated the inhibitory effect of GSH on mitosis. The mitotic indices of glutathione-fed material were consistently lower than those of control cells. Such an effect could also be observed in spruce seedlings fed with different concentrations of exogenous glutathione for different exposure times (Müller et al. 2000). In contrast to spruce tissues, in vitro cultivated *Arabidopsis* root tissue exhibited positive effects in the form of enhanced cell division upon the exogenous application of 10 to 100 µM glutathione (SÁNCHEZ-FERNÁNDEZ et al. 1997).

Furthermore, GSH treatment induced structural chromosomal defects, so called chromosomal aberrations, in the nuclei. In callus cells, these chromosomal abnormalities include stickiness, represented by sticky chromosomes and bridges, as well as vagrant chromosomes and fragments, which were found previously in experiments using young spruce seedlings (ZELLNIG et al. 2000). Chromosome stickiness may result from improper folding of chromosome fibre into single chromatids (MCGILL et al. 1974, KLASTERSKA et al. 1976).

Possibly, the proteinaceous matrix of the chromatin material is involved in producing this defect (STEPHEN 1979). The chromosome stickiness reflects intensive toxic influences (FISKESJÖ 1994), usually of an irreversible type leading to cell death (LIU et al. 1993). An increase in chromosome stickiness was observed in root tips of plants exposed to different kinds of stress (e.g. BRIAND and KAPOOR 1989, LIU et al. 1994, WIERZBICKA 1995). The other defects observed in the present study, vagrant chromosomes and fragments, are the results of genotoxic effects and can be transferred to ensuing generations of cells (FISKESJÖ 1994). These aberration types are attributed to the failure of the mitotic microtubule apparatus, also implying the involvement of protein malfunctions (BRIAND and KAPOOR 1989). As GSH is partly responsible for the conformation and redox status of protein thiol groups (KUNERT and FOYER 1993), changes in the glutathione status might also influence nuclear proteins, which could be the reason for the granular appearance of nuclei in the electron micrographs. Vagrant chromosomes and fragments will lead to an increased amount of micronuclei in the following interphase stages, which was observed upon all GSH treatments after 48 hours. Enhanced levels of micronuclei were also found in earlier experiments with GSH fed exogenously to root tips of young spruce seedlings (ZELLNIG et al. 2000). Micronuclei are used to determine the mutagenicity of chemicals and their occurrence usually reflects a highly mutagenic substance inside the cells (FARRIS et al. 1996, GIRI and KAHN 1996). The ultrastructure of micronuclei during their development in binucleate cells obtained from *Allium* is described by SANTA-CRUZ and HERVÁS (1994). The size of the micronucleus observed in the spruce callus cells with about 1.2 μm is in the reported range of 1–6 μm for *Allium* cells and the chromatin pattern is also similar.

The ultrastructural data of the spruce callus cells clearly document severe disturbances in cell organization and cell consistency upon GSH application in concentrations around 1mM. The changes were found in the cytoplasm and cell organelles such as plastids, mitochondria, and the nucleus. The massive destruction of the stroma in the plastids seems a contradiction to the current opinion that the amount of GSH inside the chloroplast stroma in living cells is thought to be close to 5 mM (NOCTOR et al. 1998). Since the externally applied GSH concentrations were far lower than 5 mM the results either indicate that an unphysiologically high amount of GSH accumulated within the plastids, or that the estimated GSH concentrations of 5 mM within plastids are far too high. Another possible explanation would be that it was not an increased plastid concentration of GSH itself that caused the disturbances, but the uptake of GSH disturbing cellular redox sensing and signalling processes that involve the GSH system (MAY et al. 1998), and that such disturbances lead to secondary effects. The internal membrane system of the plastids was obviously not affected by the GSH treatment, swelling and deterioration in the chloroplast thylakoids, as described for transgenic tobacco plants with enhanced GSH content (CREISSEN et al. 1999), could not be observed in the spruce callus cells. That indicates that the increased amount of GSH only affected stroma-bound processes.

A separation of the plasma membrane from the cell wall has been reported for plant cells in connection with toxic impacts of an increased amount of iron (REBOREDO 1997). The occurrence of small vesicles in the cytoplasm close to the plasma membrane in callus cells might be an indication of the beginning of the separation of the plasma membrane from the cell wall. The retraction of the plasma membrane could not have been induced by plasmolysis due to osmotic imbalances during fixation because the cells were fixed in an osmotically balanced solution. It has been demonstrated during protoplast isolation that

different substances can be taken up into these vesicles (cf. OPARKA 1994). Possibly, these vesicles are involved in the uptake of external GSH, but because an ultrastructural tracing method for GSH is not available yet, this question remains unsolved.

The presented data of GSH effects on plant cell tissue must also be seen in connection with other experiments with cultivated GSH-overexpressing plants, which should result in an increased stress tolerance. In this respect, CREISSEN et al. (1999) reported a paradoxical increase of oxidative stress in tobacco plants with elevated GSH-biosynthetic capacity. Exact knowledge about the effects of GSH on cellular structures is of growing importance due to its application as an anti-carcinogen and as a medical product.

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