PROLIFERATION OF PEROXISOMES IN PEA ROOT NODULES – AN INFLUENCE OF NaCl- OR Hg²⁺-STRESS CONDITIONS

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ABSTRACT

Morphometric procedures were used to examine peroxisome number and distribution in pea (*Pisum sativum* L.) root nodules under NaCl (50 mM) or HgCl₂ (7.3 µM) treatment. Peroxisomes were visualized cytochemically in meristem, invasion zone and prefixing zone of pea root nodules by catalase (EC 1.11.1.6) activity. The observations using light and electron microscopy revealed that the peroxisomes were predominantly spherical in shape and showed catalase activity. In nitrogen-fixation zone, catalase-active peroxisomes were observed occasionally. Bacteroids of nitrogen-fixing zone showed enhanced catalase activity probably as a response to higher level of oxidative stress. Fluorescence microscopy investigations revealed enhanced level of (homo)glutathione in prefixing and nitrogen-fixing zone of NaCl- and Hg²⁺-treated nodules, which served as an indicator of antioxidative response. Morphometric measurements revealed that during differentiation of meristematic cells into central tissue (bacteroidal tissue) cells an increase in peroxisome number was observed in unstressed nodules. Peroxisomes located in meristem, invasion zone and prefixing zone of NaCl- and Hg²⁺-treated nodules outnumbered that in control nodules. A substantial enlargement of peroxisome profiles was detected in NaCl- and Hg²⁺-treated nodules. Peroxisome divisions observed in meristematic and infection thread penetration zone were responsible for an increase in peroxisome number.

KEY WORDS: *Pisum sativum* L., peroxisome proliferation, peroxisome division, cytochemistry, catalase activity, morphometry, NaCl, salinity, mercury treatment, root nodules.

INTRODUCTION

Nitrogen-fixing root nodules develop as a result of symbiotic interactions between leguminous plants and rhizobia. Rhizobia enter the plant via infection threads (ITs) which direct toward nodule primordium established by a group of dividing cells in the root cortex. Bacteria released from ITs differentiate into bacteroids able to reduce atmospheric nitrogen into ammonium. High rate of nodule metabolic activity necessary for efficient nitrogen fixation (Walsh 1995) produces large amounts of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), due to the strong reducing power necessary for nitrogen fixation and the action of ferredoxin, leghemoglobin, uricase and hydrogenase (Dalton et al. 1986). Efficient H₂O₂ removal by catalases (Becana and Klucas 1992) and high level of nodule ascorbate-glutathione cycle within nodules (Matamoros et al. 1999b) prevent formation of hydroxyl radicals which can readily oxidize proteins, fatty acids and DNA (Halliwell and Gutteridge 1986).

Peroxisomes are single membrane-bound, DNA devoid organelles (Beevers 1979) which may display different

morphology and biochemical properties in different cell types of the same organism (Vaughn 1985). Peroxisomes are very dynamic organelles with regard to their shape and movement and they can bud off small peroxisome-like structures (Muench and Mullen 2003). Peroxisomes accumulate in the division plane and they participate in the formation of the cell plate probably by regulating hydrogen peroxide concentration and/or by involvement in membrane recycling (Collings et al. 2003).

Oxidative activity of peroxisomes resulting from a set of their own oxidases, leads to the production of hydrogen peroxide (H₂O₂) and superoxide radical (O₂^{·-}) (Corpas et al. 2001). Catalases and superoxide dismutases occur in both plant and bacterial cells. Catalase (EC 1.11.1.6), located in peroxisomes, scavenges cytotoxic H₂O₂ (Matamoros et al. 2003). Catalase activity of symbiotic rhizobia may serve as an indicator of nodule efficiency in nitrogen fixation process (Francis and Alexander 1972). Bacterial catalases are important for the protection of the nitrogen fixation process as lack of catalase activity in bacteroids implies their lower nitrogen fixation activity (Sigaud et al. 1999).

Both H_2O_2 and $\text{O}_2^{\cdot-}$ are scavenged by glutathione which has been found in all cell compartments including peroxisomes (Jimenez et al. 1998). Glutathione (reduced form, GSH) is a tripeptide ($\gamma\text{Glu-Cys-Gly}$) which is a major antioxidant in bacteria, animals and plants (Meister and Anderson, 1983). Bacteroids contain high level of glutathione important for protection of nitrogen fixation system from oxidative damage (Dalton et al. 1986; Moran et al. 2000). GSH induces plant defence genes that encode cell wall hydroxyproline-rich glycoproteins and phenylpropanoid biosynthetic enzymes (Wingate et al. 1988). Glutathione is involved in plant tolerance to heavy metals and NaCl (Becana and Klucas 1992; May et al. 1998). Legume plants can contain glutathione homologue, homoglutathione (hGSH: $\gamma\text{Glu-Cys-}\beta\text{Ala}$). But in opposition to soybean, bean and mungbean, glutathione instead of homoglutathione dominates in pea nodules (Matamoros et al. 1999b). (h)GSH is abundant in meristematic and bacteroidal tissue of pea nodules (Matamoros et al. 1999a). (h)GSH localization served in this work as an indicator of antioxidative defense in pea nodules treated with NaCl or Hg^{2+} .

Four types of peroxisomes have been described in plants: glyoxysomes, involved in glyoxylate cycle and gluconeogenesis; leaf peroxisomes, participating in photorespiration; nodule peroxisomes of tropical legumes engaged in ureide metabolism and unspecialized peroxisomes with undefined functions (Newcomb et al. 1985; Vaughn 1985; Mano et al. 2002). Peroxisome proliferation in plants has been reported under physiological conditions like seed germination (Mansfield and Briarty 1996) and leaf senescence (Pastori and del Río 1997). In response to external stimuli, yeast, plant and animal cells can increase the number of their peroxisomes. Chen et al. (1995) demonstrated that cadmium induced peroxisome proliferation in yeast. Palma et al. (1991) showed elevated H_2O_2 and increased peroxisome number as a response to clofibrate treatment of pea plants. Peroxisome proliferation was also demonstrated in *Lemna* as a function of light intensity (Ferreira et al. 1989). On the other hand, it was proved that catalase deficient plants were sensitive to elevated light (Willekens et al. 1997), and catalase enriched plants showed enhanced symptoms of pathogen attack (Talarczyk et al. 2002). Some xenobiotics cause dramatic proliferation of liver peroxisomes and can lead to liver cancer (Terlecky and Fransen 2000).

In animals, new peroxisomes arise as a result of divisions of the pre-existing peroxisomes or arise de novo (Terlecky and Fransen 2000). Several lines of evidence indicate that peroxisomes arise from specialized subdomain of ER (Mullen et al. 1999; Brocard et al. 2005).

The aim of this study was the comparison of peroxisome number in nodule zones under elevated stress level delivered by NaCl-salinity or mercury treatment. Peroxisome proliferation during bacteroidal tissue development was demonstrated by morphometric measurements of control as well as NaCl- and Hg^{2+} -treated pea root nodules. Cytological investigations showed that pea root nodule peroxisomes can divide.

MATERIALS AND METHODS

Plant culture

After sterilization pea seeds (*Pisum sativum* L. cv. Sześciotygodniowy) were sown in sterile perlite and grown in

a growth room at 20–22°C, with a 16 h light period (sodium lamps WLS 400W, POLAMP, Poland; light intensity 400 $\mu\text{mol/m}^2/\text{s}$ PAR). The plants were watered every three days with nitrogen-free medium according to Fahraeus (1957) and with distilled water on the remaining days. NaCl- and Hg^{2+} -treated plants were watered with the medium supplemented with 50 mM NaCl or 7.3 μM HgCl_2 , respectively. The concentrations of NaCl and HgCl_2 used in this study may be expected to produce low- to moderate-level stress symptoms concerning nodule structure and functioning (James et al. 1993; Ortega-Villasante et al. 2005). Pea plants were inoculated with *Rhizobium leguminosarum* bv viciae effective strain 248 kindly delivered by dr A.H.M. Wijffes (Institute of Molecular Plant Sciences, Clusius Laboratory, Leiden, The Netherlands). As a result of the inoculation, effective (nitrogen fixing) nodules developed on pea roots.

Cytochemical localization of catalase activity – light and transmission electron microscopy

Technique for cytochemical localization of catalase generally followed the procedure of Vaughn (1985). Longitudinal sections, ~2 mm long and less than 0.5 mm thick, of root nodules collected from 3-week old pea plants were fixed in 2% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) for 1 h at 4°C. Sections were used in order to improve 3,3'-diaminobenzidine (DAB) penetration of the tissues. Four washings in phosphate buffer (pH 7.4) were followed by a pre-incubation in 0.05 M propanediol buffer (pH 9.6) with 1 mg DAB ml^{-1} at 4°C for 30 minutes. The specimens were incubated in propanediol buffer (pH 9.6) supplemented with 0.02% (v/v) H_2O_2 at 25°C for 1 h in the dark. Pre-incubation and incubation in appropriate buffers with 100 mM 3-amino-1,2,4-triazole (catalase inhibitor) served as a control. Additional control was done without H_2O_2 in incubation medium. After four washings in 0.05 M cacodylate buffer (pH 7.2) specimens were postfixed in 1% (w/v) OsO_4 at 4°C for 2 h, dehydrated in ethanol and acetone and embedded in Epon (Luft 1961). All chemicals used in this study were purchased from SIGMA.

Longitudinal semithin sections (3 μm thick) through the nodules were stained with methylene blue and azure B and examined under bright fields of light microscopes, Axioskop or Provis (Zeiss, Germany).

Thin sections, without or with additional contrasting according to the procedure given by Reynolds (1963) were conducted by transmission electron microscopy using microscope type JEM-100C (Japan).

Fluorescence microscopy

Localization of (h)GSH was conducted on nodule hand sections stained with 4 mM monochlorobimane in 50 mM K_2HPO_4 (pH 7.0) (Dalton et al. 1998) and observed under fluorescence microscopy. Olympus Provis fluorescence microscope was equipped with U-MNU filter cube with excitation/barrier filter 360–370 nm/>420 nm.

Morphometry

Cytochemical localization of catalase together with staining of the semithin sections with methylene blue and azure B allowed visualization of peroxisomes under bright field optics. Measurements of the peroxisome number in pea nodules were conducted on semi-thin sections (3 μm thick) using ocular simple square lattice test system (5

sections from different, randomly chosen nodules per a meristem, infection thread penetration zone or young bacteroidal tissue; 8 images per section; objective 100 $\times$). The measurements followed procedures described by Weibel (1979). The number of peroxisomes was calculated per cell profile and per unit volume of a tissue. The number of peroxisomes per unit volume of a tissue (N_V) was calculated using the equation: $N_V = N_A / (D + t)$, where N_A is the number of peroxisomes per unit area of section, D is the average peroxisome diameter and t is the section thickness. D values for control as well as NaCl- or Hg^{2+} -treated peroxisomes were recalculated from data presented in Figure 9 using formula $D \approx 4/\pi \cdot d$, where d is the mean profile diameter (Weibel 1979).

Measurements of the peroxisome profile areas were performed using computerized image analyzer (AnalySiS version 3.0 of Soft Imaging System; Olympus; Japan) on the basis of electron micrographs (negative magnification 2000). About 350, randomly chosen peroxisome profile areas found in meristem and young bacteroidal tissue were measured for each control or treatments separately, and classified (Fig. 9).

Statistical analysis

The square root transformation of data was applied to peroxisome counts to achieve their normal distribution (Zar 1996). Transformed data were analysed statistically by

analysis of variance (ANOVA) for comparison of means. ANOVA was used to test for differences in peroxisome number ($P < 0.05$, Tukey test). The means, which are given in Figures 8 and 10, are expressed in terms of the original data by squaring them.

RESULTS

Zonation of pea nodules according to Vasse et al. (1990) with modification given by Hirsch (1992)

Distinct zonation of pea root nodules was observed (Fig. 1). Meristematic zone (zone I) of pea nodules was composed of small cells which possessed many small vacuoles. Divisions of meristematic cells produced new cells which differentiated into peripheral cell layers or central tissue (bacteroidal tissue) cells. According to nomenclature proposed by Hirsch (1992), peripheral cell layers could be divided into nodule cortex, nodule endodermis and nodule parenchyma. Infection threads (see Figs 1 and 6) penetrated group of cells located in the vicinity of meristematic cells, which can be named "invasion zone" (Ili zone). Release of bacteria from the infection threads produced so called "infected cells" (ic), which together with "uninfected cells" (uc) formed prefixing zone (zone II). Prefixing zone was divided into two subzones, zone IIA and zone IIB located

Fig. 1. (A) Zonation of control pea root nodule. (B) Enlarged fragment of (A).

Abbreviations: I – meristematic zone; Ili – invasion zone; IIA and IIB – subzones of the prefixing zone; II/III – interzone with large amyloplasts; IIIA – distal part of the nitrogen-fixing zone; IIIB – proximal part of the nitrogen-fixing zone; nc – nodule cortex; ne – nodule endodermis; np – nodule parenchyma; ic – infected cell; uc – uninfected cell; vb – vascular bundle; → infection thread. Scale bars: (A) 100 μ m; (B) 25 μ m.

Fig. 2. Localization of DAB-positive peroxisomes in control pea root nodule. (A) apical part of the nodule; (B) subzones IIA and IIB of the prefixing zone.

Abbreviations: I – meristematic zone; Ili – invasion zone; it – infection thread; vb – meristematic vascular bundle; IIA – distal part of zone II where bacteria are released from infection threads; IIB – proximal part of zone II; ic – infected cell; uc – uninfected cell; n – nucleus; → peroxisomes. Scale bars: 10 µm. Notice that zone IIB contains much less DAB-positive peroxisomes comparing with zone IIA.

close to zone invasion or close to so called “interzone” (zone II/III), respectively. In zone IIA bacteria were released from infection threads and differentiated into bacteroids. Infected and uninfected cells of zone IIA contained several small vacuoles. Then the vacuoles fused and usually one central vacuole was formed in each infected and uninfected cell of zone IIB. A substantial enlargement of cell size was observed during maturation of the central tissue (Fig. 1B). Interzone was distinguishable by large starch deposits and low level of cell vacuolation. Fully developed, nitrogen fixing bacteroids were characteristic for nitrogen-fixing zone (subzones IIIA and IIIB). Zone IIIB could be distinguished from zone IIIA by maximal cell size and maximal cell vacuolation (Fig. 1).

Light and electron microscopy observation of catalase-active peroxisomes

After cytochemical staining for catalase activity, peroxisomes were easily identified by light and electron microscopy (Figs 2-6). Such peroxisomes can be called “DAB-positive peroxisomes”. They were present in meristematic and both infected and uninfected cells of prefixing zone.

Peroxisomes were usually spherical in shape. After staining of semi-thin sections with methylene blue and azure B peroxisomes were visible under light microscopy as small, blue granules. The largest peroxisomes were observed in Hg^{2+} -treated nodules (compare Fig. 4 with Figs 2 and 3). In opposition to Hg^{2+} -treated nodules, DAB-positive peroxisomes gradually disappeared in zone IIB of control- and NaCl-treated nodules (compare Figs 2-4).

DAB-positive peroxisome distribution within meristem and central tissue was supported by electron microscopy (EM) observations (Fig. 5). Peroxisomes were visible in meristematic and IIA and IIB zones but were absent from zone III. They were present in both infected and uninfected cells of prefixing zone. Distinct catalase activity displayed bacteroids of zone III. Catalase activity was localized on the bacteroid surface probably referring to their periplasmic space and inside bacteroids in the form of granules or connected with membraneous structures. Bacteroids of zone III of Hg^{2+} -treated nodules were rich of DAB-positive structures in their cytoplasm (Fig. 5I). Occasionally, DAB-positive granules were observed in peribacteroidal space (Fig. 5I).

Fig. 3. Localization of DAB-positive peroxisomes in NaCl-treated nodule. (A) apical part of the nodule; (B) subzones IIA and IIB of the prefixing zone.

Abbreviations: I – meristematic zone; Ili – invasion zone; ic – infected cell; it – infection thread; n – nucleus; uc – uninfected cell; → peroxisomes; ► peroxisomes in a dividing cell. Scale bar: 10 µm.

Induction of cell divisions in the zone IIA of bacteria release from the infection threads!

Peroxisome divisions could be easily observed in meristematic zone and invasion zone of Hg^{2+} -treated nodules (Fig. 6).

Glutathione localization

Fluorescence microscopy observations revealed that GSH was located mainly in meristem, zones II/III and III as well nodule endodermis of control nodules. Within nitrogen-fixing zone, GSH was located predominantly in infected cells. NaCl-treatment resulted in a substantial increase in GSH content in meristem and nitrogen-fixing zone. Hg^{2+} -treatment resulted in increased GSH content in prefixing zone in comparison with control- and NaCl- treatments. Senescing central tissue of Hg^{2+} -treated nodules showed lower GSH content than central tissue of nitrogen-fixing zone. Hg^{2+} -treated nodules exhibited also elevated (h)GSH level in vascular boundless and nodule parenchyma (Fig. 7).

Morphometric measurements

Peroxisomes of Ili and IIA zones outnumbered that in meristem of control nodules when calculated per cell profile

or per tissue unit volume (Figs 8 and 10). The same situation was observed for NaCl-treated nodules when peroxisome number was calculated per cell profile (Fig. 8). No significant differences in peroxisome number, calculated per cell profile or tissue unit volume, were observed in meristem or central tissue zones of Hg^{2+} -treated nodules (Figs 8 and 10).

The number of peroxisomes per meristematic cell profile showed essential differences in NaCl- and Hg^{2+} -stressed nodules as compared with control (Fig. 8). The same situation was observed in invasion zone as well as prefixing zone. NaCl- and Hg^{2+} -treatments resulted in a substantial difference in peroxisome number per cell profile in meristem and zones comparing with control. The difference was especially distinct for Hg^{2+} -treated nodules in meristem and zone IIB (Fig. 8).

Peroxisome profiles areas enlarged in response to NaCl- and Hg^{2+} -treatments. The average peroxisome diameters calculated from data presented in Figure 9 were 0.936/1.15/1.38 µm for control/NaCl/ Hg^{2+} nodules, respectively.

Fig. 4. Localization of DAB-positive peroxisomes in Hg^{2+} -treated nodule. (A) meristematic zone of the nodule; (B) subzone IIB of the prefixing zone and interzone II/III located between prefixing and nitrogen-fixing zone. For more zonation see Fig. 1. Abbreviations: it – infection thread; n – nucleus; V – vacuole; $\rightarrow$ peroxisomes in meristem and young bacteroidal tissue; $\blacktriangleright$ peroxisomes in the interzone. Scale bars: 10 μm .

Notice large number of peroxisomes in zone IIB. A rapid decrease in peroxisome number is observed between zone IIB and interzone II/III.

Differences in peroxisome number calculated per tissue volume were significant between control and treatments only in meristem and zone IIB (Fig. 10).

DISCUSSION

Lopez-Huertas et al. (2000) postulate that peroxisome biogenesis is directly responsive to the stress signal H_2O_2 . Pre-existing peroxisomes enlarge by import of membrane lipids and proteins, and matrix proteins (Terlecky and Fransen 2000). Lazarow and Fujiki (1985) proposed that new peroxisomes arise by “bidding and fission” of pre-existing peroxisomes. Comparing with control plants, a substantial difference in peroxisome number was observed in nodule meristems under NaCl- or Hg^{2+} -treatments (Figs 8 and 10). This finding was supported by cytological observations which clearly indicate that peroxisomes can divide in meristematic cells (Fig. 6). However, there were no significant differences in numerical density (N_V) of peroxisomes in Iii and IIA zones independently on treatment (Fig. 10), which may result from distinct increase in cell volume observed in young bacteroidal tissue as compared with meristematic tissue (Fig. 1). But expression of peroxisome number per cell profile basis gave significant differences in peroxisome number in Iii and IIA zones between treatments (Fig. 8).

Comparing to the control, NaCl- and Hg^{2+} -treatments led to a substantial enlargement and proliferation of catalase-active peroxisomes (Figs 8-10), which was probably an adaptation of plant cells to elevated level of hydrogen peroxide. Charlton et al. (2005) showed NaCl-induced expression of peroxisome-associated genes. One can assume that an increase in oxidative stress severity caused by NaCl or Hg^{2+} resulted in an increase in catalase enzyme synthesis and its incorporation to existing peroxisomes. It can be speculated whether enlarged peroxisomes have a tendency to divide? Divisions of enlarged peroxisomes could explain differences in peroxisome number observed in meristem and Iii, IIA and IIB zones between control and treatments. But glyoxysomes of germinated cotton seeds increase substantially in volume but do not divide (Kunce et al. 1984).

Oxidative activity of peroxisomes, thanks to the presence of oxidases, leads to the production of hydrogen peroxide, which is decomposed by catalase of these organelles to water and molecular oxygen (Vaughn 1985). Catalase of plant cells is located predominantly in peroxisomes but protects the whole cells against H_2O_2 (Willekens et al. 1997). The decline in DAB-positive peroxisome number observed between zone IIB and interzone (Figs 2-4) may reflect a decrease in their oxidative activity and/or the whole plant cells due to extremely low free oxygen concentration (Walsh 1995). On the other hand, high respirato-

Fig. 5. Comparisons of DAB-positive activity in meristematic, prefixing and nitrogen-fixing zones of control as well as NaCl- or Hg^{2+} -treated pea root nodules. Meristematic, prefixing and nitrogen-fixing zones are presented in columns 1, 2 and 3, respectively. (A, B, C) control; (D, E, F) NaCl-treatment; (G, H, I) Hg^{2+} -treatment.

Abbreviations: b – bacteroid; ic – infected cell; is – intercellular space; it – infection thread; n – nucleus; uc – uninfected cell; V – vacuole; → DAB-positive peroxisome; ► DAB-positive activity inside bacteroid; ►► DAB-positive activity in peribacteroidal space; ▷ DAB-positive activity in periplasmic space of the bacteroids? Scale bars: 3 μm .

Fig. 6. DAB-positive peroxisome divisions in pea nodules treated with Hg^{2+} . (A and B) Peroxisomes divide concomitantly with cell divisions. (C) invasion zone. (D) Peroxisome division in meristematic cell.

Abbreviations: a – amyloplast; ba – bacterium inside infection thread; ch – chloroplast; cw – cell wall; G – Golgi body; it – infection thread; m – mitochondrion; V – vacuole; → peroxisome division. Scale bars: (A, B and C) 2 μm ; (D) 0.5 μm .

ry activity of bacteroids leading to the increased production of reactive oxygen species (ROS) (Puppo and Rigaud 1986) may be compensating by an increase in their own catalase activity especially of NaCl - and Hg^{2+} -stressed nodules (Fig. 5) and probably by other ROS-scavengers like glutathione (Fig. 7).

The nitrogen fixation process requires a high bacteroid respiratory rate which is accompanied by production of reactive oxygen species (ROS) (Puppo and Rigaud 1986). Additional stresses to nitrogen fixing bacteroids were delivered in the present work by NaCl - or Hg^{2+} -treatments. The fluorescence microscopy method revealed an increased level of (h)GSH in meristematic and Iii, IIA and IIB zones of NaCl - and Hg^{2+} -treated nodules. It suggests that the increase in peroxisome number and probably catalase activity observed in these tissues as the response to stress conditions was not sufficient to balance an increase in ROS content. Additional protection against ROS produced under stress conditions may be delivered by an increased level of (homo)glutathione (Fig. 7).

Zones II/III and III of the central tissue of stressed nodules also showed a higher level of (h)GSH than that of control (Fig. 7). (h)GSH was detected in infected and uninfected cells of stressed nodules (Fig. 7B and C). But, in case of zones II/III and III of control nodules, (h)GSH was detected within infected cells only (Fig. 7A). So, additional stress delivered to the nodules under NaCl or Hg^{2+} -treatment was emphasized by elevated (h)GSH level within uninfected cells.

Histochemical localization of (h)GSH in peripheral cell layers of pea nodules revealed that this thiol was located mainly in nodule endodermis under control and NaCl -treatment. But Hg^{2+} -treated nodules showed an increased level of (h)GSH in nodule parenchyma, which may protect the tissue from enhanced oxidative stress and nodule interior

from mercury entry. (h)GSH is a substrate for phytochelatase synthesis as a response to heavy metal entry into plant cells (Grill et al. 1985). On the other hand, Dalton et al. (1998) detected (h)GSH in nodule parenchyma of unstressed cowpea and bean nodules. So strict (h)GSH localization in peripheral tissues of root nodules may depend on both plant species and treatment.

Bacteroids of nitrogen-fixing zone exhibited distinct catalase activity referring to their periplasmic space and additional membraneous and granular structures observed in their cytoplasm (Fig. 5). Membraneous structures having catalase activity were especially abundant in bacteroids of Hg^{2+} -stressed nodules (Fig. 5I) and might emphasize the severity of stress. Sigaud et al. (1999) showed that catalases of *Sinorhizobium meliloti* are important during free-living growth and symbiosis with *Medicago sativa*. The importance of catalase activity was also proved during symbiosis between the luminous marine bacterium *Vibrio fischeri* and its squid host (Visick and Ruby 1998). These authors localized the catalase activity in the periplasm of the bacterium.

Peroxisome proliferation was shown in control and stressed pea root nodules by morphometric measurements and cytological investigations. DAB-positive peroxisomes were numerous in prefixing zone and were almost absent from nitrogen-fixing zone, which implied lack of oxidative metabolism within the organelle in the latter.

The level of (h)GSH in nodules increased as a response to stress delivered by NaCl or Hg^{2+} . Glutathione is probably produced by bacteroids in interzone (II/III) and nitrogen-fixing zone (zone III) of untreated nodules which suggests enhanced level of oxidative stress linked to nitrogen fixation. Additional stress was delivered by NaCl or Hg^{2+} , which resulted in an increase in (homo)glutathione level and bacteroid catalase activity.

Fig. 7. Localization of reduced (homo)glutathione in control (A), NaCl (B) and Hg²⁺ (C) treated pea root nodules under fluorescence microscopy. Abbreviations: ic – infected cell; nc – nodule cortex; np – nodule parenchyma; s – senescence zone; uc – uninfected cell; vb – vascular bundle; → nodule endodermis; for zonation see Fig. 1. Scale bars: 100 μ m.

Fig. 8. Number of DAB-positive peroxisomes per cell profile of control as well as NaCl or Hg²⁺ treated nodules. Abbreviations: I – meristematic zone; Ili – invasion zone; IIA – distal part of the prefixing zone; IIB – proximal part of the prefixing zone. For zonation see Fig. 1. Different letters indicate significant differences in mean values for each zone (P<0.05).

Fig. 9. Distribution of DAB-positive peroxisome profile areas in the prefixing zone of control as well as NaCl or Hg²⁺ treated nodules.

Fig. 10. Number of DAB-positive peroxisomes per 1 mm³ of meristematic (zone I) or young central tissue (zones Ili, IIA and IIB) of control as well as NaCl or Hg²⁺ treated pea root nodules (×10⁶). For zonation see Fig. 1. Different letters indicate significant differences in mean values for each zone (P<0.05).

To the authors knowledge this is the first report which documents plant peroxisome divisions by EM studies and an increase of peroxisome number during cell differentiation of unstressed as well as NaCl- or Hg²⁺-stressed nodules.

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LITERATURE CITED

- BECANA M., KLUCAS R.V. 1992. Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence. *Proc. Natl. Acad. Sci. USA* 89: 8958-8962.
- BEEVERS H. 1979. Microbodies in higher plants. *Ann. Rev. Plant Physiol.* 30: 159-193.
- BROCARD C.B., BOUCHER K.K., JEDESZKO C., KIM P.K., WALTON P.A. 2005. Requirement for microtubules and dynein motors in the earliest stages of peroxisome biogenesis. *Traffic* 6: 386-395.
- CHARLTON W.L., MATSUI K., JOHNSON B., GRAHAM I.A., OHME-TAKAGI M., BAKER A. 2005. Salt-induced expression of peroxisome-associated genes requires components of the ethylene, jasmonate and abscisic acid signaling pathways. *Plant Cell. Envir.* 28: 513-524.
- CHEN T., LI W., SCHULZ P.J., FURST A., CHIEN P.K. 1995. Induction of peroxisome proliferation and increase of catalase activity in yeast, *Candida albicans*, by cadmium. *Biol. Trace Element Res.* 50: 125-133.
- COLLINGS D.A., HARPER J.D.I., VAUGHN K.C. 2003. The association of peroxisomes with the developing cell plate in dividing onion root cells depends on actin microfilaments and myosin. *Planta* 218: 204-216.
- CORPAS F.J., BARROSO J.B., DEL RÍO L.A. 2001. Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends Plant Sci.* 6: 145-150.
- DALTON D.A., JOYNER S.L., BECANA M., ITURBE-ORMAETXE I., CHATFIELD J.M. 1998. Antioxidant defenses in the peripheral cell layers of legume root nodules. *Plant Physiol.* 116: 37-43.
- DALTON D.A., RUSSEL S.A., HANUS F.J., PASCOE G.A., EVANS H.J. 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc. Natl. Acad. Sci. USA* 83: 3811-3815.
- FAHRAEUS G. 1957. The infection of clover root hairs by nodule bacteria studied by a single glass slide techniques. *J. Gen. Microbiol.* 16: 374-381.
- FERREIRA R.M.B., BIRD B., DAVIES D.D. 1989. The effect of light on the ultrastructure and organization of *Lemna* peroxisomes. *J. Exp. Bot.* 40: 1029-1035.
- FRANCIS A.J., ALEXANDER M. 1972. Catalase activity and nitrogen fixation in legume root nodules. *Can. J. Microbiol.* 18: 861-864.
- GRILL E., WINNACKER E.-L., ZENK M.H. 1998. Phytochelatins: the principle heavy-metal complexing peptides of higher plants. *Science* 230: 674-676.
- HALLIWELL B., GUTTERIDGE J.M.C. 1986. Oxygen free radicals and iron in relation to biology and medicine. Some problems and concepts. *Arch. Biochem. Biophys.* 246: 501-514.
- HIRSCH A.M. 1992. Developmental biology of legume nodulation. *New Phytol.* 122: 211-237.
- JAMES E.K., SPRENT J.I., HAY G.T., MINCHIN F.R. 1993. The effect of irradiance on the recovery of soybean nodules from sodium chloride-induced senescence. *J. Exp. Bot.* 44: 997-1005.
- JIMENEZ A., HERNANDEZ J.A., PASTORI G., DEL RÍO L.A., SEVILLA F. 1998. Role of ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. *Plant Physiol.* 118: 1327-1335.
- KUNCE C.M., TRELEASE R.N., DOMAN D.C. 1984. Ontogeny of glyoxysomes in maturing and germinated cotton seeds – a morphometric analysis. *Planta* 161: 156-164.
- LAZAROW P.B., FUJIKI Y. 1985. Biogenesis of peroxisomes. *Ann. Rev. Cell Biol.* 1: 489-530.
- LOPEZ-HUERTAS E., CHARLTON W.L., JOHNSON B., GRAHAM I.A., BAKER A. 2000. Stress induces peroxisome biogenesis genes. *EMBO J.* 19: 6770-6777.
- LUFT J.M. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9: 409.
- MANO S., NAKAMORI C., HAYASHI M., KATO A., KONDO M., NISHIMURA M. 2002. Distribution and characterization of peroxisomes in *Arabidopsis* by visualization with GFP: dynamic morphology and actin-dependent movement. *Plant Cell Physiol.* 43: 331-341.
- MANSFIELD S.G., BRIARTY L.G. 1996. The dynamics of seedling and cotyledon cell development in *Arabidopsis thaliana* during reserve mobilization. *Int. J. Plant Sci.* 157: 280-295.
- MATAMOROS M.A., BAIRD L.M., ESCUREDO P.R., DALTON D.A., MINCHIN F.R., ITURBE-ORMAETXE I., RUBIO M.C., MORAN J.F., GORDON A.J., BECANA M. 1999a. Stress-induced legume root nodule senescence. Physiological, biochemical, and structural alterations. *Plant Physiol.* 121: 97-111.
- MATAMOROS M.A., DALTON D.A., RAMOS J., CLEMENTE M.R., RUBIO M.C., BECANA M. 2003. Biochemistry and molecular biology of antioxidants in the rhizobia-legume symbiosis. *Plant Physiol.* 133: 499-509.
- MATAMOROS M.A., MORAN J.F., ITURBE-ORMAETXE I., RUBIO M.C., BECANA M. 1999b. Glutathione and homogluthathione synthesis in legume root nodules. *Plant Physiol.* 121: 879-888.
- MAY M.J., VERNOUX T., LEAVER C., VAN MONTAGU M., INZÉ D. 1998. Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J. Exp. Bot.* 49: 649-667.
- MEISTER A., ANDERSON M.E. 1983. Glutathione. *Ann. Rev. Biochem.* 52: 711-760.
- MORAN J.F., ITURBE-ORMAETXE I., MATAMOROS M.A., RUBIO M.C., CLEMENTE M.R., BREWIN N.J., BECANA M. 2000. Glutathione and homogluthathione synthetases of legume nodules: cloning, expression, and subcellular localization. *Plant Physiol.* 124: 1381-1392.
- MUENCH D.G., MULLEN R.T. 2003. Peroxisome dynamics in plant cells: a role for the cytoskeleton. *Plant Sci.* 164: 307-315.
- MULLEN R.T., LISENBEE C.S., MIERNYK J.A., TRELEASE R.N. 1999. Peroxisomal membrane ascorbate peroxidase is sorted to a membranous network that resembles a subdomain of the endoplasmic reticulum. *Plant Cell* 11: 2167-2185.
- NEWCOMB E.H., TANDON S.R., KOWAL R.R. 1985. Ultrastructural specialization for ureide production in uninfected cells of soybean root nodules. *Protoplasma* 125: 1-12.
- ORTEGA-VILLASANTE C., RELLÁN-ÁLVAREZ R., DEL CAMPO F.F., CARPENA-RUIZ R.O., HERNÁNDEZ L.E. 2005. Cellular damage induced by cadmium and mercury in *Medicago sativa*. *J. Exp. Bot.* 56: 2239-2251.
- PALMA J.M., GARRIDO M., RODRÍGUEZ-GARCÍA M., DEL RÍO L.A. 1991. Peroxisome proliferation and oxidative stress mediated by activated oxygen species in plant peroxisomes. *Archiv Biochem. Biophys.* 287: 68-74.
- PASTORI G.M., DEL RÍO L.A. 1997. Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes. *Plant Physiol.* 113: 411-418.

- PUPPO A., RIGAUD J. 1986. Superoxide dismutase: an essential role in the protection of the nitrogen fixation process? FEBS Lett. 201: 187-189.
- REYNOLDS E.S. 1963. The use of lead citrate at high pH as an electron opaque in electron microscopy. J. Cell Biol. 17: 208-213.
- SIGAUD S., BECQUET V., FRENDON P., PUPPO A., HEROUART D. 1999. Differential regulation of two divergent *Sinorhizobium meliloti* genes for HPII-like catalases during free-living growth and protective role of both catalases during symbiosis. J. Bacteriol. 181: 2634-2639.
- TALARCZYK A., KRZYMOWSKA M., BORUCKI W., HENNING J. 2002. Effects of yeast CTA1 gene expression on response of tobacco plants to tobacco mosaic virus infection. Plant Physiol. 129: 1032-1044.
- TERLECKY S.R., FRANSEN M. 2000. How peroxisomes arise. Traffic 1: 465-473.
- VASSE J., DE BILLY F., CAMUT S., TRUCHET G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. 172: 4295-4306.
- VAUGHN K.C. 1985. Structural and cytochemical characterization of three specialized peroxisome types in soybean. Plant. 64: 1-12.
- VISICK K.L., RUBY E.G. 1998. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. J. Bacteriol. 180: 2087-2092.
- WALSH K.B. 1995. Physiology of the legume nodule and its response to stress. Soil Biol. Biochem. 27: 637-655.
- WEIBEL E.R. 1979. Stereological methods – practical methods for biological morphometry, vol. 1. Academic Press, New York London.
- WILLEKENS H.S., CHAMNONGPOL S., DAVEY M., SCHRAUDNER M., LANGEBAEELS C., VAN MONTAGU M., INZE D., VAN CAMP W. 1997. Catalase is a sink for H₂O₂ and is indispensable for stress defense in C-3 plants. EMBO J. 16: 4806-4816.
- WINGATE V.P.M., LAWTON M.A., LAMB C.J. 1988. Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol. 87: 206-210.
- ZAR J.H. 1996. Biostatistical analysis. Prentice-Hall International, INC., pp. 279-281.