Effects of salicylic acid on the structure of second leaves of *Hordeum vulgare* L.

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Abstract

The structure and ultrastructure of the second leaves of 10-d-old barley plants (*Hordeum vulgare* L. cv. Alfa) was investigated after long-term treatment with salicylic acid (SA) in concentrations of 0.1, 0.5 and 1.0 mM. The treatment induced: *1*) suppressed bulliform cells formation in the adaxial epidermis (1.0 mM SA); *2*) reduction of apoplast in the mesophyll (0.5 and 1.0 mM SA); *3*) formation of invaginations (0.1 and 0.5 mM SA) and proliferations (0.5 mM SA); and *4*) thylakoid destruction and coagulation of the stroma (1.0 mM SA).

Additional key words: barley, adaxial and abaxial epidermis, mesophyll, stroma, thylakoid, chloroplasts.

Introduction

Exogeneous application of salicylic acid (SA) to plants exerts diverse physiological effects, such as inhibition of dry mass accumulation (Schettel and Balke 1983), promotion of stomatal closure (Larque-Saavedra 1979), control of ion uptake and transport (Harper and Balke 1981), and inhibition of ethylene synthesis (Leslie and Romani 1986). Evidence for the involvement of SA in induction of an alternative respiratory pathway (Elthon *et al.* 1989) and expression of a nuclear gene encoding the alternative oxidase protein in *Sauromantum guttatum* (Rhoads and McIntosh 1991) has been presented. In recent investigations we have demonstrated that treatment of barley seedlings with SA decreased the rate of photosynthesis, stomatal conductance and the carboxylating activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, and increased the CO_2 compensation concentration (Pancheva *et al.* 1996, Pancheva and Popova 1998). The identified functional deviations could have structural analogues on different organization levels. Thus the aim of this study was structural analysis of the architecture of leaves and organization of the plastid apparatus of barley after 7-d treatment with different SA concentrations.

Materials and methods

Plants: Seeds of barley (*Hordeum vulgare* L., cv. Alfa) were germinated for 2 d in two layers of moist filter paper in moist vermiculite at 25 °C in the dark. Seedlings were grown in Petri dishes containing 40 cm^3 distilled water or equal amounts of SA solutions (concentrations 0.1, 0.5 and 1.0 mM), pH 4.6 - 4.8. Stock of SA (*Sigma*, St. Louis, USA) was prepared in a small volume of ethanol (final concentration 1 %), diluted to its final concentration in water and kept refrigerated until use. The solutions were changed every 24 h. Plants were grown on SA solutions for 7 d. The growing conditions were: temperature 25 ± 2 °C, relative humidity 60 ± 5 %, 12-h photoperiod, and irradiance 160 mol m^{-2} s^{-1} PAR ("White" fluorescent lamps). All measurements were done on the second fully expanded leaves of 10-d-old seedlings.

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Abbreviations: SA - salicylic acid; SEM - scanning electron microscopy; TEM - transmission electron microscopy.

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Light microscopy: Segments from the middle parts of the second leaf were fixed in 3 % glutaraldehyde (pH 7.4) and used for light microscopy *Amplival 4* microscope (*Carl Zeiss*, Jena, Germany) studies. Cross sections were cut by hand. The thickness of the lamina between the bundles, the adaxial and abaxial epidermis, the mesophyll, and the distance between bundles were measured. Ten morphometric measurements were repeated in triplicate.

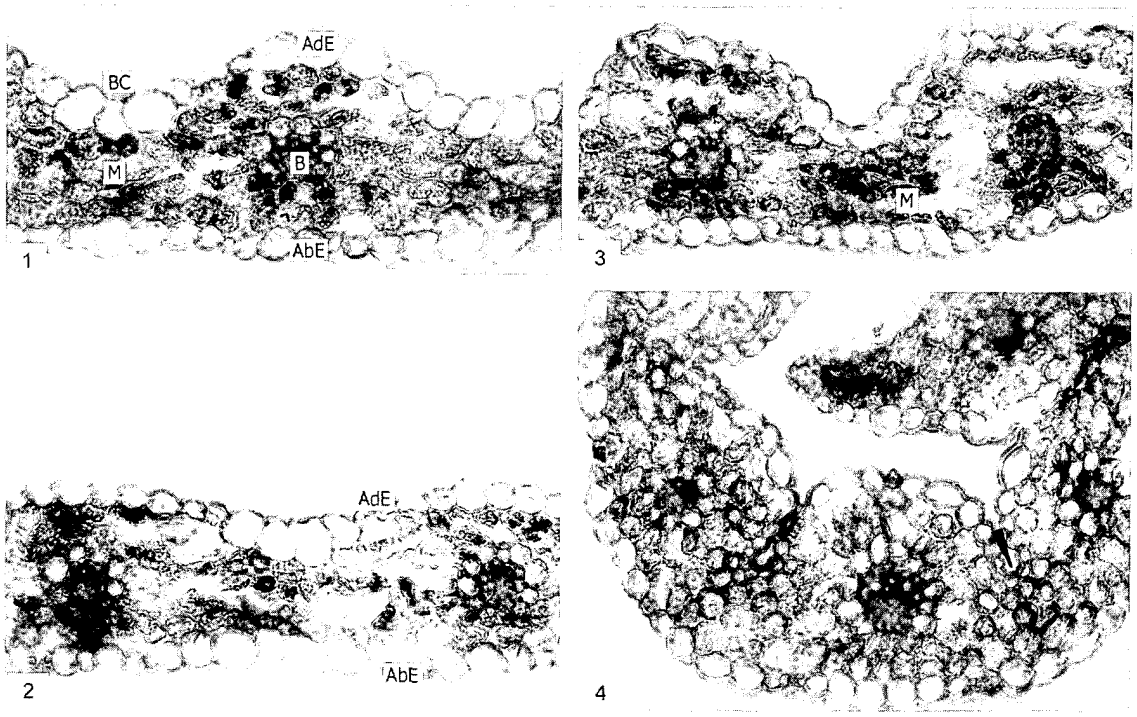
Transmission electron microscopy (TEM): For electron microscopy studies material was taken from the middle parts of lamina of the second leaf. The material was fixed in 3 % phosphate buffered glutaraldehyde (pH 7.4) for 12 h at 4 °C and postfixed in 2 % OsO_4 for 4 h at room temperature. After dehydration (increasing concentrations of ethyl alcohol - from 25 to 100 %) was embedded in *Durcupan* (*Fluka,* Switzerland) and cut with *Tesla* (Prague, Czech Republic) ultramicrotome. Examination was performed with *JEOL 1200 EX* (Tokyo, Japan) electron microscope.

Scanning electron microscopy (SEM): The segments of 0.75 mm were taken from the middle parts of lamina and were fixed in 3 % phosphate buffered glutaraldehyde (pH 7.4) for 12 h at 4 °C and postfixed in 2 % OsO_4 for 4 h at room temperature. Then the leaf tissue was dehydrated in ethyl alcohol and propylene oxide (*Serva,* Germany). Final dehydration was carried out with dehydrating appartus at critical point of evaporation with amyl acetate (*Sigma*) and liquid CO_2. The samples were covered with 0.1 nm golden layer in vacuum-evaporator *JEE-4B JEOL* and observed by using a scanning electron microscope *JSM-35 JEOL* at cathode voltage of 15 kV.

Results and discussion

Leaf structure: The second leaf of barley is unifacial, amphistomatic, typically festucoid (Fig. 1). The epidermal cells of adaxial epidermis between the vascular bundles are bulliform, in a fan-shaped group (from 4 - 5 cells). The thickness of lamina and adaxial epidermis (bulliform cells) in second leaf of 10-d-old plants were 39.10 ± 3.17 μm and 12.10 ± 1.16 μm, respectively (Table 1). The adaxial epidermis comprised 31 % of the total lamina thickness. The abaxial epidermis occupied smaller share (24 %). The mesophyll is structured of similarly arranged from chlorenchyma cells in both leaf sides. The thickness of mesophyll was 18.40 ± 2.54 μm.

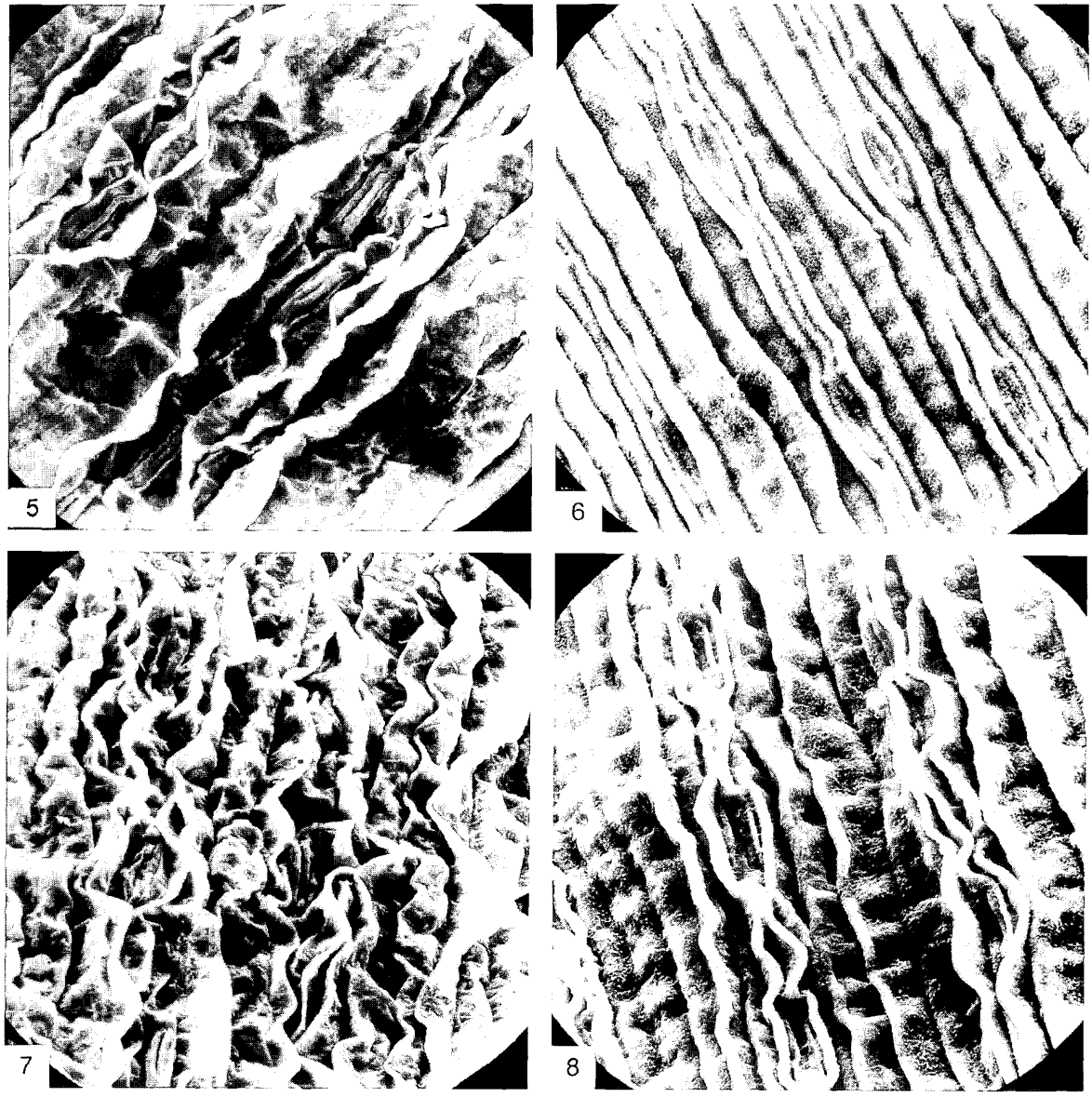
After 7-d treatment with 0.1, 0.5 and 1.0 mM SA, the microscopic analysis revealed significant deviations in



Figs. 1 - 4. Anatomical structure of second leaf in 10-d-old control barley plants (*1*) and after treatment with SA in concentrations of 0.1 mM (*2*), 0.5 mM (*3*) and 1.0 mM (*4*) [× 160]. AdE - adaxial epidermis, AbE - abaxial epidermis, BC - bulliform cells, M - mesophyll, B - bundle.

Table 1. The thickness [μm] of leaf lamina (between bundles) and its tissues in second leaf of barley plants after 7-d SA treatment.

SA [mM]	Lamina	Adaxial epidermis	Abaxial epidermis	Mesophyll	Distance between bundles
0 (control)	39.10 ± 3.17	12.10 ± 1.16	9.20 ± 1.25	18.40 ± 2.54	100.20 ± 8.27
0.1	33.60 ± 5.69	8.30 ± 1.14	8.30 ± 1.14	16.80 ± 3.99	84.30 ± 6.93
0.5	28.40 ± 2.92	6.30 ± 1.25	6.30 ± 0.98	15.40 ± 3.02	74.30 ± 8.38
1.0	26.60 ± 3.20	4.00 ± 0.89	7.80 ± 1.66	14.30 ± 1.89	62.30 ± 7.97



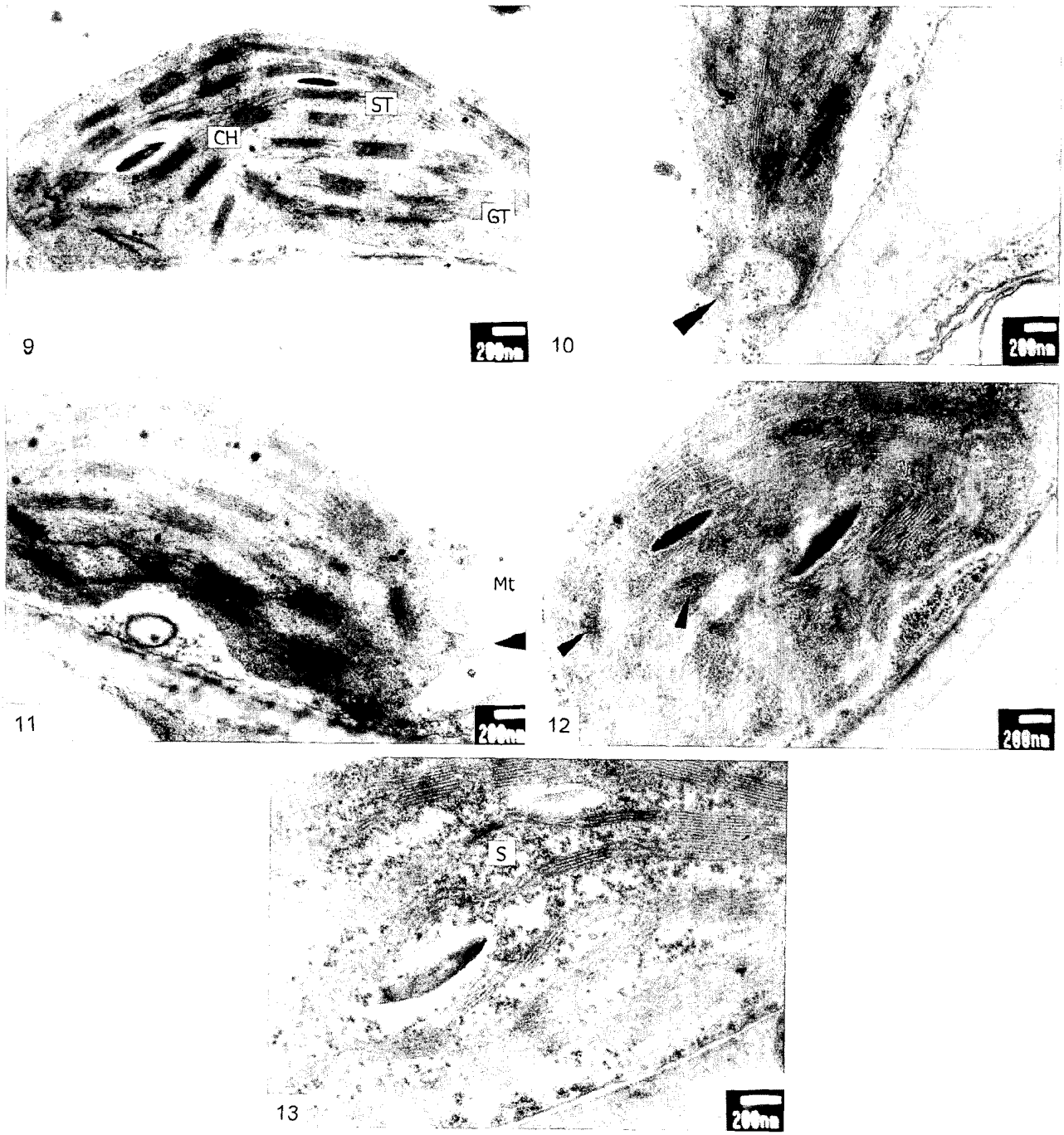
Figs. 5 - 8. Adaxial cuticle (*5*) and abaxial cuticle (*6*) in control plants and after treatment with 1.0 mM SA (*7*, *8*) (SEM) [× 400].

the leaf structure. At a concentration of 0.1 mM the average thickness of lamina between the vascular bundles was by 8 % lesser (Table 1) and mesophyll was thinner than in the control plants. It accounted for about 50 % of lamina thickness and under treatment claimed a greater share than the one identified for mesophyll in the control plants. The height of bulliform cells was reduced and accounted for 25 % of lamina thickness in the treated plants. The cells of both epidermis had similar morphological characteristics (Fig. 2). Morphometric data

(Table 1) showed the same thickness of abaxial epidermis, but less than in the controls. This testified to significant deviations in the development of bulliform cells. A similar trends of were registered in plants treated with 0.5 mM SA (Fig. 3). Uniform reduction of thickness was observed in all tissues, as well as in the average lamina thickness (Table 1). Adaxial and abaxial epidermes accounted each for 23 %, and mesophyll for 54 % of the leaf thickness. The lesser mesophyll thickness in treated plants was related to partial reduction of intercellular spaces. This is typical for the leaves of plants under stress. It was observed in leguminous plants after treatment with simulated acid rain (Stoyanova and Velikova 1998) and with copper (Barceló *at al.* 1988, Maksymiec *et al.* 1995). 1.0 mM SA induced folding of leaves and reduction of their adaxial surface (Fig. 4). Distance between the vascular bundles was twice smaller than in the control plants (Table 1). This was due to the very small size of bulliform cells. The average height of bulliform cells was 4.00 ± 0.89 μm. It was nearly three times less than as in the control plants and accounted only for 15 % of general lamina thickness of the treated plants. The average thickness of abaxial epidermis was almost twice adaxial emidermis. SA predominantly affected the bulliform cells of epidermal tissue, probably suppressing their normal development. More data on the leaf morphology of plants treated with SA were obtained by SEM analysis. It showed significant structural changes in the cuticle of plants treated with 1.0 mM SA. As compared to the adaxial and abaxial cuticles of the control plants (Figs. 5, 6), the cuticle under treatment was strongly creased (Figs. 7, 8). The change was more pronounced in the adaxial cuticle (Fig. 7). Entirely affected was the parallel orientation of cuticle creases, probably owing to changes in the leaf architecture. Less pronounced creasing was observed in the abaxial cuticle, where cuticle creases had preserved their parallel structure (Fig. 8).

Ultrastructure of chloroplasts: The mesophyll of the second leaves of barley plants was a completely differentiated photosynthesizing tissue. The chloroplasts had a well developed inner membrane system (Fig. 9). It consisted of regularly arranged grana stacks and a well developed system of intergranal membranes. In plants subjected to 0.1 mM SA the chloroplast structure was close to that of the control plants. Membrane-bound areas of lower electron density than stroma were present in some chloroplasts (Fig. 10, arrow). They were formed by invagination of the chloroplast envelope and enclosure of portions of cytosol. Invaginations were registered in the leaves of *Raphanus sativus* L. as a reaction of chloroplasts to treatment with abscisic acid (Colquhoun *et al.* 1975). The authors linked the invaginations in the stroma with activation of the hydrolytic enzymes. Invaginations resulting from SA treatment were probably due to structural changes in the membrane of chloroplasts. At 0.5 mM SA, the chloroplasts which have already formed invaginations, developed also short proliferations which enclosed some mitochondria (Fig. 11, *arrow*). Comparatively extensive associated regions were formed between the organelles. Proliferations were also observed in the mesophyll of leaves of *Ranunculus glacialis* as a structural adaptation for creation of active transport mechanisms (Lutz 1987). They were typical for species with a short life-time in the alpine summer period. Increase in contact area between the chloroplasts and mitochondria was related to the enhanced role of mitochondrial component in the cell energy system. Probably as the result of functional deviations the chloroplasts underwent structural transformations with a compensatory effect, without significant changes in the inner membrane system. Long-term treatment of plants with 1.0 mM SA induced two types of deviations from the typical chloroplast structure (Figs. 12, 13). In some mesophyll cells the chloroplasts showed obscure dilatation of stroma thylakoids and peripheral grana thylakoids (Fig. 12). Similar reaction of the membrane system was observed in chloroplasts of the same species after treatment with jasmonic acid in concentrations of 25 and 250 μM (Popova and Uzunova 1996). Crystal-like structures close to stroma centres were formed in the stroma of the same chloroplasts (Fig. 12, *arrow*). Such formations were observed by Ascaso and Rapsch (1986) after treatment of *Quercus rotundifolia* seedlings with evernic acid. Baszynski *et al.* (1988) and Maksymiec *et al.* (1995) registered them in spinach and bean leaves after the treatment of plants with copper. They characterized them as phytoferritin particles formed after the onset of destructive changes in the membrane system. Ouzounidou *et al.* (1997) considered them as aggregations of microtubule-like structures, after observing them in the chloroplasts of both untreated and Cd-treated wheat plants. They were related to development of plastids and transformations in the inner membrane system. The fact that they had not been observed in the control plants testified that they were SA-induced and were probably of a membrane origin. In mesophyll cells close to adaxial epidermis the chloroplast membrane was destroyed and the stroma coagulated (Fig. 13).

The results of our study have shown that 0.5 and 1.0 mM SA exercises a significant impact on the structural organization of photosynthetic apparatus of barley plants. SA suppresses the formation of bulliform cells in adaxial epidermis and modifies their function. It does not influence significantly the mesophyll structure, but changes the ultrastructure of chloroplasts.



Figs. 9 - 13. Ultrastructure of chloroplasts in control plants (*9*) and after treatment with 0.1 mM (*10*), 0.5 mM (*11*), and 1.0 mM SA (*12, 13*). CH - chloroplast, GT - grana thylakoid, ST - stroma thylakoid, S - stroma, MT - mitochondria.

References

Ascaso, C., Rapsch, S.: Ultrastructural changes in chloroplasts of *Quercus rotundifolia* Lam. in response to evernic acid. - Ann. Bot. **57**: 407-413, 1986.

Barcelo, J., Vazquez, M.D., Poschenrieder, C.: Structural and ultrastructural disorders in cadmium-treated bush bean plants (*Phaseolus vulgaris* L.). - New Phytol. **108**: 37-49, 1988.

Baszynski, T., Tukendorf, A., Ruszkowska, M., Skorzynska, E., Maksymiec, W.: Characteristics of the photosynthetic apparatus of copper non-tolerant spinach exposed to excess

copper. - J. Plant Physiol. **132**: 708-713, 1988.

Colquhoun, A.J., Hillman, L.R., Crewe, C., Bowes, B.G.: An ultrastructural study of the effects of abscisic acid on senescence of leaves of radish (*Raphanus sativus* L.). - Protoplasma **84**: 205-221, 1975.

Elthon, T.E., Nickels, R.L., McIntosh, L.: Mitochondrial events during the development of thermogenesis in *Sauromantum gutatum* (Schett.). - Planta **180**: 82-89, 1989.

Harper, J.R., Balke, N.E.: Characterisation of the inhibition of K^+ absorption in oats roots by salicylic acid. - Plant Physiol. **68**: 1349-1353, 1981.

Larque-Saavedra, A.: Stomatal closure in response to acetylsalicylic acid treatments. - Z. Pfanzenphysiol. **93**: 371-375, 1979.

Leslie, C., Romani, R.: Salicylic acid: a new inhibitor of ethylene biosynthesis. - Plant Cell Rep. **5**: 144-146, 1986.

Lutz, C.: Cytology of high alpine plants. II. Microbody activity in leaves of *Ranunculus glacialis* L. - Cytologia **52**: 679-686, 1987.

Maksymiec, W., Bednara, J., Baszyński, T.: Responses of runner bean plants to excess copper as a function of plant growth stages: Effects on morphology and structure of primary leaves and their chloroplast ultrastructure. - Photosynthetica **31**: 427-435, 1995.

Ouzounidou, G., Moustakas, M., Eleftheriou, E.P.: Physiological and ultrastructural effects of cadmium on wheat (*Triticum aestivum* L.) leaves. - Arch. Environ. Contam. Toxicol. **32**: 154-160, 1997.

Pancheva, T.V., Popova, L.P., Uzunova, A.N.: Effects of salicylic acid on growth and photosynthesis in barley plants. - J. Plant Physiol. **149**: 57-63, 1996.

Pancheva, T.V., Popova, L.P.: Effect of salicylic acid on the synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase in barley leaves. - Plant Physiol. **152**: 381-386, 1998.

Popova, L.P., Uzunova, A.N.: Changes in the chloroplast ultrastructure of barley leaves under treatment with jasmonic acid. - Photosynthetica **32**: 635-639, 1996.

Rhoads, D.M., McIntosh, L.: Isolation and characterization of a cDNA clone encoding an alternative oxidase protein in *Sauromantum guttatum* (Schett.). - Proc. nat. Acad. Sci. USA **88**: 2122-2126, 1991.

Schettel, N.L., Balke, N.E.: Plant growth response to several allelopathic chemicals. - Weed Sci. **31**: 293-298, 1983.

Stoyanova, D., Velikova, V.: Effects of simulated acid rain on chloroplast ultrastructure of primary leaves of *Phaseolus vulgaris* L. - Biol. Plant. **40**: 589-595, 1998.