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Apoptosis in Wheat Seedlings Grown under Normal Daylight

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Abstract—Apoptosis was observed in the coleoptile and initial leaf in 5-8-day-old wheat seedlings grown under normal daylight. Apoptosis is an obligatory event in early wheat plant ontogenesis, and it is characterized by cytoplasmic structural reorganization and fragmentation, in particular, with the appearance in vacuoles of specific vesicles containing intact organelles, chromatin condensation and margination in the nucleus, and internucleosomal fragmentation of nuclear DNA. The earliest signs of programmed cell death (PCD) were observed in the cytoplasm, but the elements of apoptotic degradation in the nucleus appeared later. Nuclear DNA fragmentation was detected after chromatin condensation and the appearance in vacuoles of specific vesicles containing mitochondria. Two PCD varieties were observed in the initial leaf of 5-day-old seedlings grown under normal daylight: a proper apoptosis and vacuolar collapse. On the contrary, PCD in coleoptiles under various growing (light) conditions and in the initial leaf of etiolated seedlings is only a classical plant apoptosis. Therefore, various tissue-specific and light-dependent PCD forms do exist in plants. Amounts of O_2^- and H_2O_2 evolved by seedlings grown under normal daylight are less than that evolved by etiolated seedlings. The amount of H_2O_2 formed in the presence of sodium salicylate or azide by seedlings grown under normal daylight was increased. Contrary to etiolated seedlings, the antioxidant BHT (ionol) did not inhibit O_2^- formation and apoptosis and it had no influence on ontogenesis in the seedlings grown under normal daylight. Thus, in plants grown under the normal light regime the powerful system controlling the balance between formation and inactivation of reactive oxygen species (ROS) does exist and it effectively functions. This system is responsible for maintenance of cell homeostasis, and it regulates the crucial ROS level controlling plant growth and development. In etiolated plants, this system seems to be absent, or it is much less effective.

Key words: antioxidant, apoptosis, cytoplasm fragmentation, coleoptile, hydrogen peroxide, ontogenesis, plants, wheat

Development of etiolated wheat seedlings is accompanied by programmed cell death (PCD) in the coleoptile and initial leaf [1-9]. In these organs, individual signs of apoptosis were detected already in 3-6-day-old seedlings, and in 8-day-old plants, they are expressed very strongly [4, 8]. The ultrastructural features of apoptosis in etiolated wheat seedlings are the condensation of cytoplasm in the apoptotic cells, the appearance in them of myelin-like structures, the formation of huge vacuoles and the specific fragmentation of cytoplasm, the appearance in vacuoles of specific vesicles containing active organelles, the condensation and margination of chromatin in the nucleus, and the internucleosomal fragmentation of nuclear DNA [4, 5, 8, 9]. Margination of chromatin (uneven distribution and displacement of chromatin in the nucleus) is due to hydrolysis of proteins that are responsible for chromatin binding to the nuclear envelope. The single-membrane vesicles observed in vacuoles of apoptotic cells of the initial leaf [8, 9] look similar to vacuolar vesicles detected in apoptotic coleoptile cells [6], but they contain mainly plastids [8, 9] but not mitochondria as in the coleoptile. It seems that all these apoptotic events in the nucleus and cytoplasm are well coordinated, but their sequence is still unclear. Some data show that apoptotic degradation of cytoplasm precedes the destruction of the nucleus; on the other hand, there are also data that clear-cut destructive changes in the nucleus may appear earlier than real initial apoptosis signs in cytoplasm [4]. According to a classification [10] there are three types of PCD in plants: apoptosis-like cell death, cell death similar to dying of aging cells in a leaf, and cell death that is

Abbreviations: BHT) butylated hydroxytoluene or 2,6-di-*tert*-butyl-4-methylphenol (ionol); PCD) programmed cell death; ROS) reactive oxygen species.

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due to vacuolar collapse. It is yet unknown whether all these three PCD types can appear in wheat plants.

Apoptosis in plants is controlled by reactive oxygen species (ROS) [4]; it is known that cyclic formation of superoxide-anion ($O_2^{\cdot-}$) is essential to the development of etiolated wheat seedlings [11]. In etiolated seedlings, it is inhibited by the known antioxidant butylated hydroxytoluene (BHT or ionol) [11]. In these seedlings, BHT ($2.27 \cdot 10^{-4}$ M) completely prevents apoptosis in coleoptile [4, 8], but peroxides induce apoptosis in the leaf [9] and strongly stimulate it in the coleoptile [9]. Unlike the strong BHT geroprotector action in coleoptile, this antioxidant in the initial leaf did not block apoptotic DNA fragmentation and it did not prevent the aged formation of specific vacuolar vesicles containing subcellular organelles [9]. It was assumed that such a different action of BHT in the leaf and coleoptile of etiolated wheat seedling may be due to higher oxygen concentration in the leaf than that in the coleoptile [9], when BHT loses its antioxidant properties and may turn out to be a $O_2^{\cdot-}$ producer [12].

There is no doubt that the etiolated seedling is very interesting, but it is still an artificial plant model. Unfortunately, it is still unknown whether apoptosis and what kinds of PCD in general proceed in the wheat seedlings under natural plant growth conditions. Therefore, it was important to learn when apoptosis starts, how it proceeds, and what are the features of apoptosis in wheat plants grown under normal daylight and how the antioxidant BHT influences apoptosis under these particular conditions.

Some biochemical (internucleosomal DNA fragmentation) and ultrastructural features of apoptosis in the coleoptile and initial leaf in combination with measurements of $O_2^{\cdot-}$ and H_2O_2 formed in wheat seedlings of various age grown under normal daylight in the presence or absence of BHT were investigated in the present work.

MATERIALS AND METHODS

Seedlings of Mironovskaya 808 variety winter wheat (*Triticum aestivum* L.) were grown in a climatic chamber (Fision, England) at 24°C and under conditions of normal daylight duration (12 h long) in plastic cuvettes on filter paper, the edges of which were immersed into water (control) or BHT (ionol) solution ($2.27 \cdot 10^{-4}$ M). BHT solution and water were changed once a day. To prepare BHT solution (50 mg/liter) the stock solution of BHT in ethanol (50 mg/ml) was added to boiling water and then the weakly opalescent BHT solution obtained ($2.27 \cdot 10^{-4}$ M) was cooled down to room temperature. The equivalent ethanol volume was added also to water used for growing control seedlings.

Seedlings of defined age (the seedling age was estimated in days starting from the beginning of the seed soaking time) were thoroughly washed with water; and coleoptiles and initial leaves were separated and used for DNA isolation or fixed for electron-microscopy analysis. Each experiment was done at least twice and accompanied with an independent control (plant growing on water under the same conditions).

To isolate DNA, the coleoptiles or leaves were thoroughly ground in a mortar with pestle in liquid nitrogen, a lysing solution (50 mM Tris-HCl, pH 7.5, 25 mM EDTA, 1% SDS) was added to the fine powder obtained, and the mixture was then incubated for 30 min at room temperature. Then NaCl was added to 1 M concentration, and the mixture was deproteinized by careful shaking with chloroform–isoamyl alcohol mixture (10 : 1 v/v). After centrifugation for 10 min at 5000*g*, DNA was precipitated from the aqueous phase by addition of three volumes of 96% ethanol and dissolved then in 50 mM Tris-HCl-buffer, pH 7.5, containing 25 mM EDTA. The DNA obtained was treated with DNase-free ribonuclease A (50 μg/ml) for 20 min at 37°C, and DNA was precipitated again with addition of three volumes of 96% ethanol.

Similar aliquots of isolated DNA preparations were electrophoresed for 2 h in 1.2% agarose gels at 2-3 V/cm in 0.09 M Tris-borate buffer, pH 8.3, containing 0.5 μg/ml ethidium bromide.

Intact seedlings were fixed for electron microscopic analysis in 25% glutaraldehyde and 2% OsO_4 solution. Seedlings were cut from the seeds; coleoptile and leaf were separated at 0°C in a fixing solution prepared in 0.1 M buffer, pH 7.2 (0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4). Twenty milliliters of the phosphate buffer, 300 mg sucrose, 3 ml 25% glutaraldehyde, and 0.5 ml 40% formaldehyde were used for preparation of the fixing solution. After vacuum treatment, the material was left in fixing solution at room temperature for 1.5-2 h. Then the fixing solution was poured off and the material was incubated for 30 min in freshly prepared buffer, and the samples were then incubated in 2% OsO_4 solution for 1.5 h (second fixation). The material was then dehydrated with ethanol (increasing concentrations). The fixed material was stored at 2°C. After dehydration the sections were placed into acetone–Epon 812 mixture with gradual increase in the Epon 812 concentration (2 : 1, incubation for 3 h; 1 : 1, incubation for 24 h; 1 : 2, incubation for 24 h at room temperature). The material was finally placed in Epon 812 and incubated for 24 h at 37°C and then for 5 days at 60°C.

Sections obtained with an LKB-III Microtome (Sweden) were stained according to Reynolds and analyzed with Hitachi-11 or Hitachi-12 electron microscopes (Japan).

We measured amount of $O_2^{\cdot-}$ evolved into the water phase by two intact seedlings immersed fully into phosphate buffer, pH 7.8 (4 ml). $O_2^{\cdot-}$ formed by seedlings was registered in the water phase using the trap nitrotetrazolium blue (Sigma, USA) as described earlier [11]. The

incubation period of seedlings in the medium with nitrotetrazolium blue was 1 h. Incubation was performed in a darkness at 26°C. To enhance the emergence of $O_2^{\cdot-}$ from seedling into medium, Triton X-100 (Fluka, USA; 0.1%) was added to the incubation medium.

The specificity of $O_2^{\cdot-}$ determination was achieved using two parallel samples in each separate experiment. Superoxide dismutase (Sigma, 50 units/ml medium) was added to one of these samples, this blocking fully the reduction of nitrotetrazolium blue due to the effective elimination of $O_2^{\cdot-}$. The amount of $O_2^{\cdot-}$ formed was determined by the difference between parallel samples in absorption values at 530 nm of reduced nitrotetrazolium blue measured with a Hitachi 557 spectrophotometer (Japan).

To measure amount of H_2O_2 formed by one seedling, it was washed with bidistilled water, dried with filter paper and placed into "counting solution" containing 3 mM Tris-HCl buffer (pH 8.75) + $15 \cdot 10^{-5}$ M *p*-iodophenol + $15 \cdot 10^{-5}$ M luminol + 0.1 nM horseradish peroxidase (total volume was 930 μl).

To investigate the influence of salicylate or azide on the H_2O_2 formation, a seedling was immersed for 1 min in 10 ml 2 mM sodium salicylate or 0.1 mM sodium azide in bidistilled water, pH 5.85, then it was washed with bidistilled water, dried with filter paper, placed into "counting solution", and H_2O_2 amount was measured. All these manipulations were done under dim light.

RESULTS AND DISCUSSION

Ultrastructural features of apoptosis in wheat seedlings. In wheat seedlings grown under conditions of 12-h-long daylight, apoptosis was observed in the coleoptile and initial leaf similarly to that detected earlier in etiolated wheat seedlings [4, 5, 8, 9]. This programmed event is an obligatory element in plant development, and it is realized in about similar period of seedling life being relatively independent of light conditions (light, darkness) of plant growth. Similarly to plants grown in darkness [4, 5, 8, 9], in seedlings grown under normal daylight the apoptotic internucleosomal DNA fragmentation in coleoptiles begins on the sixth day of the seedling life, and in 7-8-day-old seedlings it is much stronger (Fig. 1a). Under normal daylight, the apoptosis in the initial leaf seems to begin earlier than that in etiolated seedlings. For example, in seedlings grown in the light the apoptotic DNA fragmentation (Fig. 1a) and marked condensation and margination of chromatin in apoptotic cells (Fig. 2) in the initial leaf were observed already on the fifth day of the seedling life, whereas the apoptotic DNA fragmentation in the leaf of etiolated seedlings was detected only in 8-day-old plants [4, 5, 8, 9]. Probably this acceleration of the appearance of apoptosis is associated with ROS formation due to photosynthesis in the green leaf.

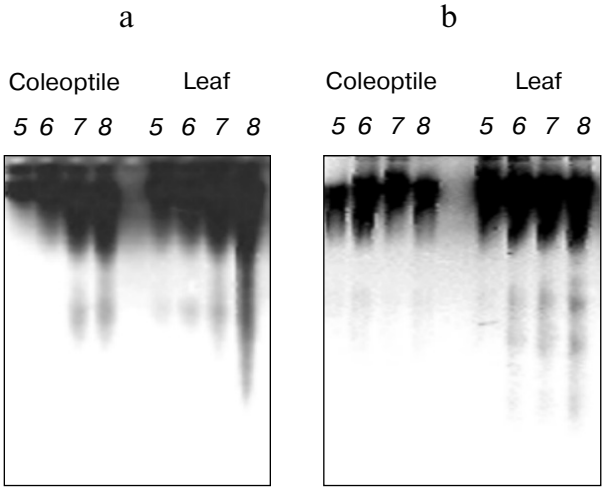

Fig. 1. Electrophoregrams of DNA from initial leaves and coleoptiles of wheat seedlings grown under 12-h-long daylight: a) control plants; b) plants grown in the presence of $2.27 \cdot 10^{-4}$ M BHT. Numerals indicate the seedling age (days).

Along with the mentioned apoptotic changes in the nucleus, specific structural changes in cytoplasm (condensation and fragmentation of cytoplasm and an appearance of a huge vacuole) were also observed in the cells of the initial leaf of 5-day-old wheat seedlings grown under normal daylight (Fig. 3). These apoptotic changes in the cytoplasm are similar to that observed earlier in apoptotic cells of etiolated seedlings. The cells of the apical zone of the initial leaf are mainly similar in ultrastructure with parenchymal cells of the coleoptile apical zone (Figs. 3 and 4). A large central vacuole and a narrow wall-located tonoplast layer are specific for most of these cells. The cytoplasm of these typical apoptotic cells does not have any signs of destruction, and it contains many elements of Golgi apparatus, endoplasmic reticulum, and the all main cell organelles—nucleus, mitochondria, plastids, and ribosomes. Plastids in the cells of the apical leaf zone are represented mainly by chloroplasts with a well developed thylakoid system, but in coleoptile cells by amyloplasts and leucoplasts. In the cells of the apical zones of the initial leaf and coleoptile, the condensation and margination of chromatin in the nuclei were often observed (Fig. 2). As in etiolated plants, in seedlings grown under normal daylight specific cytoplasmic vesicles containing mitochondria (Fig. 4b) and plastids (Fig. 4c) were observed in vacuoles of parenchymal apoptotic cells of the apical zone of the coleoptile. Very similar vesicles with intense mitochondrial DNA synthesis and containing mitochondria actively consuming oxygen were first detected by us in apoptotic cells of the initial leaf and the coleoptile in etiolated wheat seedlings; these vesicles were first described by us and even isolated as a separate organelle fraction [6].

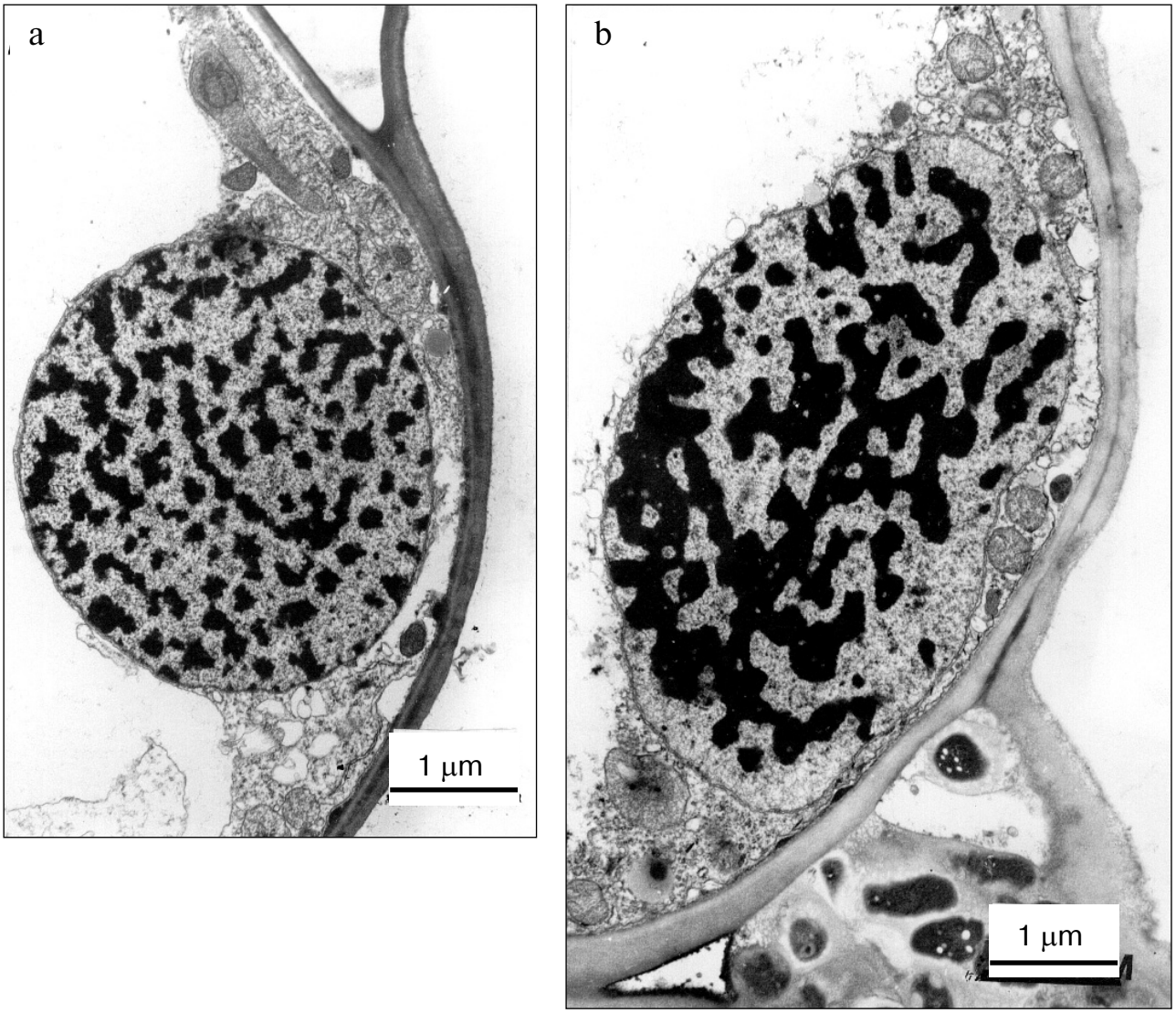

Fig. 2. Nuclei in the parenchymal cells of the apical zone of the initial leaf in a 5-day-old wheat seedling: a) nucleus with relatively even distribution of decondensed chromatin; b) nucleus with marked condensed and marginated (displaced) chromatin.

Thus, like in etiolated seedlings, the apoptosis in the coleoptile and initial leaf of seedlings grown under normal daylight is accompanied by: 1) formation in the tissue of apoptotic centers—individual cells with electron-dense cytoplasm and organelles; 2) structural reorganization and fragmentation of cytoplasm and, in particular, with an appearance in it of specific single-membrane vesicles containing intact active organelles; 3) condensation and margination of chromatin in the cell nucleus; and 4) internucleosomal fragmentation of nuclear DNA. In other words, the apoptosis in various tissues in etiolated and green seedlings proceeds in a similar way, and its ultrastructural features described seem to be universal for apoptosis in plants. Apoptosis does not critically depend on the light conditions during wheat plant growth, and it is an obligatory programmed element of early ontogenesis of the wheat plant.

As mentioned already in the introduction, the programmed cell death (PCD) in plants can be classified into three types [10]. The ultrastructural features of PCD in the coleoptile and initial leaf in etiolated wheat seedlings as well as in seedlings grown under normal daylight showed that PCD in these plant organs is represented mainly by apoptosis. The earliest PCD signs we observed in cytoplasm, but the elements of apoptotic degradation in the nucleus appeared later. In any case, we have detected the fragmentation of nuclear DNA already after chromatin condensation and the appearance in vacuoles of specific cytoplasmic vesicles containing mitochondria.

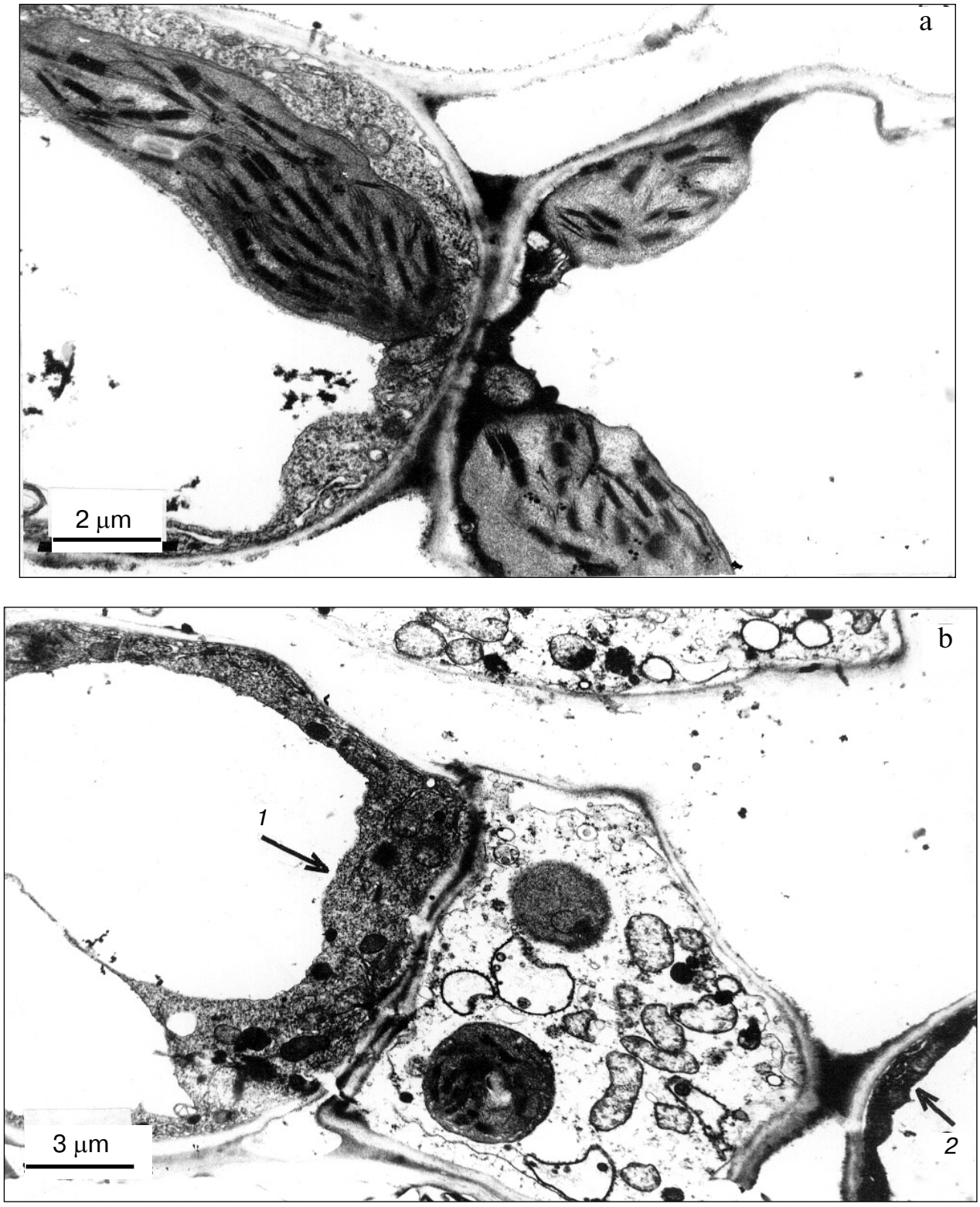

Fig. 3. Fragments of parenchymal cells in the apical zone of the initial leaf of a 5-day-old wheat seedling. a) Fragment of the dark electron-dense apoptotic cell (on the right) that neighbors a cell with normal structure. b) Parenchymal cell at a stage of the vacuolar collapse, it neighbors being normal cells containing electron-dense mitochondria (*1*) and dark electron-dense apoptotic cell (*2*).

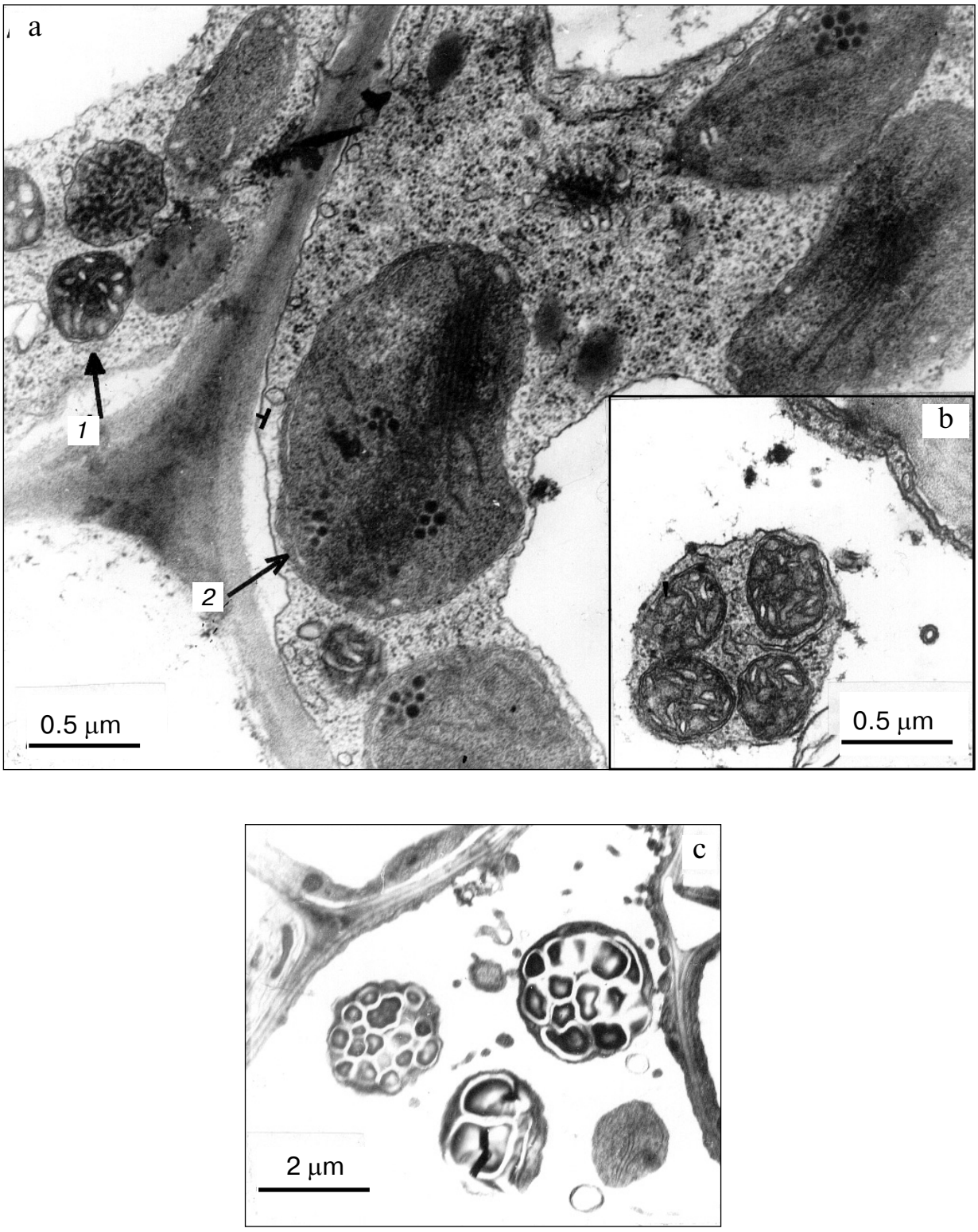

Fig. 4. Ultrastructure of parenchymal cells in the apical zone of the initial leaf of a 5-day-old wheat seedling. a) The protoplast regions in parenchymal cells of the coleoptile apical zone. Arrows show a mitochondrion (*1*) and plastid (*2*). b) Cytoplasmic vesicle containing mitochondria in a vacuole of the coleoptile cell. c) Vacuolar vesicles containing plastids (amyloplasts).

It is known that PCD in aging plant leaves proceeds both by apoptosis [13] and another slower death mechanism [14]. In cells dying by slow death, the fragmentation of the nucleus and the formation of the central vacuole occur after full degradation of chloroplasts, and these events are the terminal stages of apoptosis. In this particular case, the protoplast degrades slowly and some cell ingredients are translocated into other developing plant organs [14].

In the parenchymal tissue of the apical zone of the initial leaf of wheat seedlings grown under normal daylight, along with cells having normal structure and cells with ultrastructural apoptotic features, we have detected cells that degrade by a third PCD mechanism (by vacuolar collapse) (Fig. 3b) but not by the mechanism of early plastid degradation. It was noticed that plastids are first to die during leaf aging [14]. This is not the case in the wheat initial leaf. On the contrary, plastids in the leaf cells are the most stable subcellular organelles; they are still observed when the nucleus and mitochondria are already destroyed (Fig. 3b).

PCD with central vacuole involvement is most specific for xylogenesis but it may appear also during aging of the generative organs in higher plants [15]. It was suggested that vacuolar collapse *in vivo* in the cells of tracheal elements in plants is due to changes in tonoplast permeability to organic ions. The vacuolar collapse is accompanied with liberation from the vacuole of proteases and nucleases that attack the cell organelles. The central vacuole swells, and it occupies most of the volume of the differentiating tracheal cell [16]. Vacuolar collapse is a corrugation and fragmentation of the central vacuole [17]. The cytoplasm current stops in a few minutes after the vacuole content is mixed with cytoplasm, and all cellular organelles are subjected to degradation. Organelles with a single membrane (endoplasmic reticulum, Golgi apparatus) are first to be destroyed, and then the organelles with two membranes (mitochondria, chloroplasts) are degraded; in the beginning their matrix is destroyed, the organelles swell, and then the integrity of their external and internal membranes is damaged. It is assumed that auotophagy takes place during degradation of cellular organelles and autolysis of cells of the tracheal elements. The typical apoptotic features, such as chromatin condensation and nuclear fragmentation, were not observed in this PCD [16].

A cell in the late stage of vacuolar collapse can be seen in the apical zone of the initial leaf in a 5-day-old wheat seedling (Fig. 3b); this cell neighbors a cell with normal structure. Contrary to current ideas on the priority of the plastid degradation during leaf aging [14], in the cell observed in the initial leaf of a wheat seedling the chloroplasts among all cellular organelles are most preserved but their structure is significantly changed, practically all other organelles being already destroyed. In the initial leaf cells in 5-day-old seedlings the mitochondria are also seen but they are strongly degraded, practically do not have cristae, and their matrix is strongly enriched with water (Fig. 3b).

Thus, two PCD varieties going in parallel were observed in the initial leaf of the 5-day-old wheat seedlings grown under normal daylight, i.e., the proper apoptosis and the cell death by vacuolar collapse. PCD in the coleoptiles of wheat seedlings grown under various light conditions and PCD in the initial leaf of etiolated seedlings are classical apoptosis. Thus, the tissue-and light-dependent PCD form has specificity in plants.

The antioxidant BHT does not prevent the apoptosis under normal daylight. The growth of etiolated wheat seedlings is strongly inhibited in the presence of BHT ($0.45 \cdot 10^{-5}$-$2.27 \cdot 10^{-4}$ M), the morphology of their organs is changed, and the lifespan of the coleoptile is markedly increased [8, 18]. In the coleoptile BHT prevents the decrease in total DNA and protein contents with age [11], the apoptotic internucleosomal fragmentation of nuclear DNA, and the appearance in cellular vacuoles of specific vesicles with active mitochondria replicating DNA and the synthesis of heavy mitochondrial DNA (ρ = 1.718 g/cm^3) [8]. Besides, BHT induces structural changes in organization of all cellular organelles (nucleus, mitochondria, plastids, Golgi apparatus, and endoplasmic reticulum) and the formation of new unusual membrane structures in cytoplasm. BHT distorts the division of nuclei and cells. This results in the appearance of large multiblade polyploid nuclei and multinuclear cells. This action of BHT is due to its antioxidative properties (the structural BHT analog 3,5-di-*tert*-butyltoluene is physiologically inert and does not have these properties). It was assumed that ROS inactivation by BHT is indeed responsible for a prevention of apoptosis and the distortion of mitosis in etiolated wheat seedlings [8].

In contrast, BHT at the same concentration ($2.27 \cdot 10^{-4}$ M) practically does not inhibit the growth of wheat seedlings grown under normal daylight (seedlings do not differ morphologically from the control plants).

In contrast to etiolated seedlings [8], BHT does not prevent apoptotic DNA fragmentation in the coleoptile of plants grown under normal daylight. On growth in the presence of BHT under normal daylight the apoptotic DNA fragmentation in coleoptile, like in control (grown without BHT) etiolated plants [8], was observed in the 6-8-day-old seedlings (Fig. 1b) but it seems to be less strong than that in control etiolated seedlings [8]. In the initial leaf of seedlings grown under normal daylight BHT does not prevent apoptosis (DNA fragmentation) and it seems even to slightly stimulate it (Fig. 1b). To some extent this corresponds to our ultrastructural investigations: in contrast to etiolated plants [8], BHT does not change the ultrastructure of cells of parenchymal tissues in coleoptile and initial leaf of seedlings grown under normal daylight.

A vacuole with vesicles containing subcellular organelles appears in apoptotic cells and the margination and condensation of chromatin were observed in the nuclei in plants grown in the presence of BHT under normal daylight, similarly to control (grown without BHT) seedlings (photographs are not given as they are practically identical with those of control seedlings grown without BHT under normal daylight). It was shown earlier that in etiolated wheat seedlings BHT prevents the appearance of the ultrastructural apoptosis signs in the cells of coleoptile but not in the initial leaf [9]. The cytoplasmic vesicles containing mitochondria in cellular vacuoles in the initial leaf of both etiolated seedlings [9] and seedlings grown under normal daylight seem to appear even more often in plants grown in the presence of BHT than that in control plants grown without BHT. Therefore, judging from the ultrastructural features, unlike in etiolated plants [8], BHT may even slightly speed up the appearance of apoptosis in the coleoptile parenchymal cells when plants are grown under normal daylight [9].

The different character of the action of BHT in various tissues and depending on the growth light conditions seems to be due to different oxygen content in the cells. It is known that BHT and its analogs can manifest prooxidant properties at increased oxygen partial pressure: BHT interacts with O_2 with formation of $O_2^{\cdot-}$ [12]. BHT is oxidized by a single-electron mechanism into carcinogenic BHT-quinone that is able to generate $O_2^{\cdot-}$ under appropriate conditions [12, 19]. As far as in darkness the oxygen content in plant tissues is significantly decreased, the reaction of the BHT single-electron oxidation is diminished, this being most likely responsible for the antioxidative action of BHT on the coleoptile cells in etiolated seedlings.

ROS formation by seedlings. Formation of reactive oxygen species (ROS) accompanies ontogenesis and it is a physiologically essential element of plant development because, in particular, ROS control mitosis and apoptosis [4, 11]. In a large set of experiments with etiolated wheat seedlings, we earlier established that superoxide-anion ($O_2^{\cdot-}$) forms intensively in them and it evolves into the incubation medium. We do not know yet exactly what kind of particular biochemical system takes part directly in the $O_2^{\cdot-}$ formation under our experimental conditions. But because the lifetime of $O_2^{\cdot-}$ is very short, we suggest that namely $O_2^{\cdot-}$ formed on the external cytoplasmic membrane of the cells in the surface plant tissues is liberated into the water phase. The most common source of $O_2^{\cdot-}$ in plasmatic membrane is NADPH-oxidase.

The rate of the $O_2^{\cdot-}$ formation by seedlings is not constant; it increases rapidly at the early stages of development of etiolated seedling, and it is maximal on the third-fourth day of the seedling life [11]. The pattern of the cyclic changes in the rate of the $O_2^{\cdot-}$ formation in the surface tissues of etiolated seedling showed the existence of a correlation between plant growth and its ability to generate $O_2^{\cdot-}$. BHT (a trap of free radicals) added to the cultivation medium decreased the ability of etiolated seedlings to form $O_2^{\cdot-}$ [11].

In the present work, we have shown that a system of regulation of ROS level is induced in seedlings grown under normal daylight; there is no such system in etiolated seedlings. The rate of the $O_2^{\cdot-}$ formation in a plant in the light should be increased due to water decomposition. Our experiments showed that in plants grown under normal daylight the rate of $O_2^{\cdot-}$ formation and the level of H_2O_2 do not increase, but they are even lower than that in etiolated seedlings (Figs. 5 and 6). The results of the experiments with wheat seedlings grown in the presence of the $O_2^{\cdot-}$ trap (BHT) under normal daylight also turned out to be unexpected. Under these conditions, unlike in etiolated seedlings, BHT did not inhibit the ability of the seedling to generate $O_2^{\cdot-}$ (Fig. 7), and it did not distort the seedling growth and development (seedlings grown in the presence of BHT under normal daylight did not differ morphologically from the control plants).

These data along with results of previous experiments [11] indicate that plants grown under normal daylight have a qualitatively different system of ROS synthesis/decay compared with etiolated seedlings. It should be mentioned that an active ROS-neutralizing system dependent on the plant growth condition has already been described. For example, the superoxide dismutase

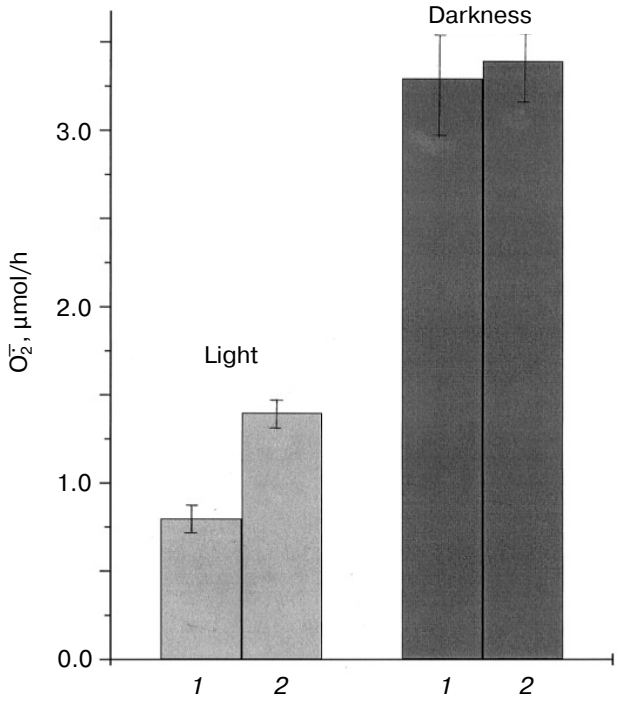

Fig. 5. Amount of $O_2^{\cdot-}$ evolved in 1 h into the incubation medium by 6- (*1*) and 7-day-old (*2*) wheat seedlings grown under normal daylight and in darkness.

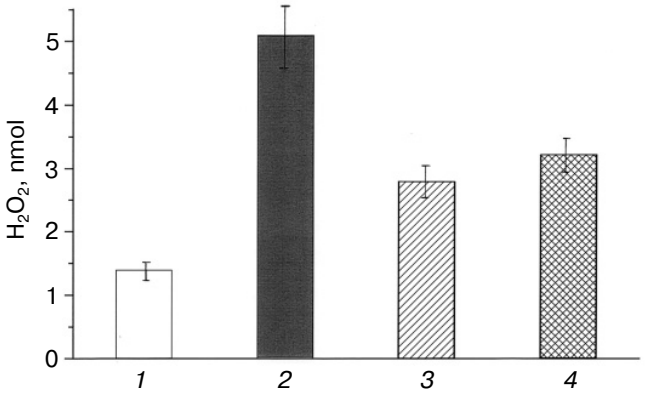

Fig. 6. Amount of H_2O_2 evolved in 1 min into the incubation medium by 6-day-old wheat seedlings grown under normal daylight (*1*) and in darkness (*2*) or incubated on the light in the presence of sodium salicylate (*3*) or azide (*4*) added to the incubation medium.

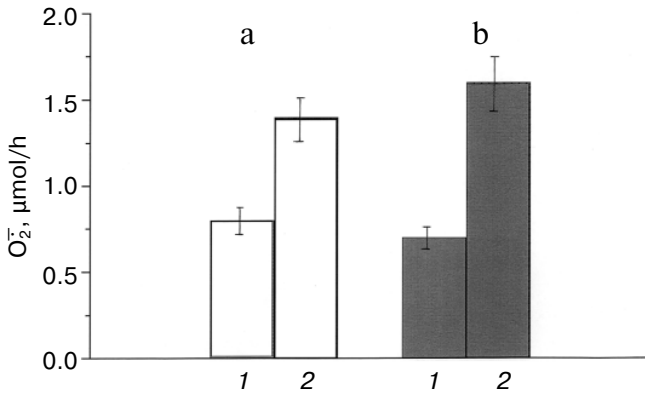

Fig. 7. Amount of O_2^- evolved in 1 h into the incubation medium by 6- (*1*) and 7-day-old (*2*) wheat seedlings grown under normal daylight in the absence (a) or presence of $2.3 \cdot 10^{-4}$ M BHT (b).

(SOD) activity in barley leaves is decreased in darkness [20], but in the light (wheat plants) the SOD activity is strongly increased due to the *de novo* enzyme synthesis induced by the light [21].

We assumed that in wheat seedlings grown under normal daylight the cascade of the enzymatic reactions catalyzing the effective inactivation of O_2^- formed under oxygen excess in the plant (formation of H_2O_2 from O_2^- by SOD and decomposition of H_2O_2 by catalase) is activated. To verify this scheme, we performed special experiments using inhibitors of catalase activity (sodium salicylate and azide). Addition of these inhibitors to seedlings grown under normal daylight before the H_2O_2 is measured results in an increase in H_2O_2 level (Fig. 6). In contrast, in etiolated seedlings neither of the catalase inhibitors influenced the H_2O_2 level (results not given). This shows that in seedlings grown in darkness the conjugated system of ROS neutralizing reactions does not play an essential role.

Thus, our experiments show that in seedlings grown under normal daylight the system of relatively rigid regulation of the ROS level functions effectively. On one hand, this system prevents excessive O_2^- accumulation and, on other hand, it does not permit the ROS level to be lower than the critical level necessary for plant development. There is no such system in etiolated seedlings or it is very ineffective. As mentioned already, in the light the oxygen content in plant tissues is increased as result of the decomposition of water. This may result in an increase in the O_2^- generation rate. But this effect correlates with light-induced activation of the ROS content regulating system observed. The physiological role of this system may be very essential as an increased ROS level can activate apoptosis. In fact, apoptosis was observed when the catalase inhibitor salicylic acid was added [4]; according to our data, salicylic acid increases the H_2O_2 level. This correlation seems to be nonrandom as an increase in the oxygen level can increase the rate of the ROS generation in tissues and, therefore, activate apoptosis.

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