Biochemistry (Moscow), Vol. 66, No. 8, 2001, pp. 850-859. Translated from Biokhimiya, Vol. 66, No. 8, 2001, pp. 1048-1059.
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Effect of the Antioxidant Ionol (BHT) on Growth and Development of Etiolated Wheat Seedlings: Control of Apoptosis, Cell Division, Organelle Ultrastructure, and Plastid Differentiation

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Received October 23, 2000
Revision received March 6, 2001

Abstract—Ionol (BHT), a compound having antioxidant activity, at concentrations in the range 1-50 mg/liter ($0.45 \cdot 10^{-5}$-$2.27 \cdot 10^{-4}$ M), inhibits growth of etiolated wheat seedlings, changes the morphology of their organs, prolongs the coleoptile life span, and prevents the appearance of specific features of aging and apoptosis in plants. In particular, BHT prevents the age-dependent decrease in total DNA content, apoptotic internucleosomal fragmentation of nuclear DNA, appearance in the cell vacuole of specific vesicles with active mitochondria intensively producing mtDNA, and formation of heavy mitochondrial DNA (ρ = 1.718 g/cm^3) in coleoptiles of etiolated wheat seedlings. BHT induces large structural changes in the organization of all cellular organelles (nucleus, mitochondria, plastids, Golgi apparatus, endocytoplasmic reticulum) and the formation of new unusual membrane structures in the cytoplasm. BHT distorts the division of nuclei and cells, and this results in the appearance of multi-bladed polyploid nuclei and multinuclear cells. In roots of etiolated wheat seedlings, BHT induces intensive synthesis of pigments, presumably carotenoids, and the differentiation of plastids with formation of chloro- or chromoplasts. The observed multiple effects of BHT are due to its antioxidative properties (the structural BHT analog 3,5-di-*tert*-butyltoluene is physiologically inert; it has no effect similar to that of BHT). Therefore, the reactive oxygen species (ROS) controlled by BHT seem to trigger apoptosis and the structural reorganization of the cytoplasm in the apoptotic cell with formation of specific vacuolar vesicles that contain active mitochondria intensively producing mtDNA. Thus, the inactivation of ROS by BHT may be responsible for the observed changes in the structure of all the mentioned cellular organelles. This corresponds to the idea that ROS control apoptosis and mitosis including formation of cell wall, and they are powerful secondary messengers that regulate differentiation of plastids and the Golgi apparatus in plants.

Key words: antioxidant, apoptosis, BHT, carotenoids, cell ultrastructure, chloroplast, chromoplast, DNA fragmentation, DNA synthesis, mitochondria, mitochondrial DNA, mitosis, ontogenesis, plastids, plastid differentiation, PCD, plant, ROS, wheat

Plants are constantly subjected to oxidative stress due, in particular, to photosynthesis, a powerful oxygen-producing process. For example, the concentration of molecular oxygen in leaves may reach up to about 250 μM, and a significant part of this is present as reactive oxygen species (ROS) [1]. Along with their destructive effect, ROS may have very important signal functions in the cell: they may control the cell cycle and trigger programmed cell death (PCD) in plants [2, 3]. For example, H_2O_2 serves as a main signal of regulation of the cell cycle and PCD in plants; it stimulates a rapid Ca^{2+} inflow into cells and plays the role of a secondary proapoptotic signal [4].

We found that in etiolated wheat seedlings a cyclic formation of superoxide takes place [5], and this process coincides with the DNA replication cycles in the coleoptile and initial leaf. It was suggested that formation of superoxide is crucial for seedling development, and it seems to control plant growth by cell elongation [5].

On the other hand, plants possess various effective systems that protect them from oxidative stress by inactivating ROS that are constantly formed [6, 7]. In particular, plants are usually characterized by high content of such antioxidants as α-tocopherol, ascorbic acid, glutathione, carotenoids, flavones, various polyphenolic

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

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compounds, and other compounds. Nevertheless, the mechanisms of functioning of the antioxidative systems in plants are very poorly studied both *in vitro* and *in vivo*. This particularly concerns the effects of antioxidants on growth and development of intact plants. The effects of antioxidants on genetic processes and molecular mechanisms of cellular differentiation in plants are practically not investigated. At the same time, it is well known that in animals the antioxidants and effective ROS scavengers such as BHT act as powerful geroprotectors [8-11].

Along with anti-mutagenic action [12], BHT may distort mitosis, and this is accompanied by the appearance of multinuclear cells in plants [13]. We have observed that BHT strongly inhibits the growth of etiolated wheat seedlings, and it seems also to influence plastid differentiation [14]. Thus, in fact, the antioxidants may control plant growth and cell differentiation, and they are involved in regulation of various genetic processes in the plant cell. Nevertheless, the molecular mechanisms of these effects of antioxidants on ROS are still poorly investigated, and the biochemical and cytological status of the cell under the influence of antioxidants inactivating ROS is unclear.

Considering the important role of ROS as secondary messengers, we have tried to determine whether the antioxidant BHT can influence: 1) the development of etiolated wheat seedlings; 2) the synthesis of DNA and PCD in coleoptiles of intact etiolated wheat seedlings of various age, and 3) the ultrastructure of different cellular organelles (nucleus, plastids, mitochondria, Golgi apparatus) and the plastid differentiation.

MATERIALS AND METHODS

Seeds of Mironovskaya 808 variety of winter wheat (*Triticum aestivum* L.) were germinated in darkness for 24 h at 26°C on wet filter paper in a plastic cuvette; sprouted seeds were transferred into other cuvette, covered with a lid, and grown for 24 h in darkness at 26°C. Then, for the experimental plants, water as the medium was changed for BHT solution (Sigma, USA; 1-50 mg/liter, $0.45 \cdot 10^{-5}$-$2.27 \cdot 10^{-4}$ M) or 3,5-di-*tert*-butyltoluene or ascorbic acid (Aldrich, USA), and the growth of the seedlings was continued in darkness at 26°C for a few days. Thus, the whole plant was exposed to the compounds used and their effects were judged from analysis of the separated plant organs (coleoptile, initial leaf). The reagent solutions used were changed once a day for freshly prepared solutions. To prepare BHT water solution, solid BHT was dissolved in ethanol and added to boiling water to the concentration required, then the weakly opalescent mixture was cooled down to room temperature. An equivalent volume of ethanol was added to the water used for growing the control wheat seedlings.

Seedlings of defined age (seedling age was estimated in days starting from the beginning of the seed soaking time) were thoroughly washed with water; coleoptiles, initial leaves, and roots were separated and used for determination of the DNA or protein content. Each experiment (plant growing in the presence of each compound tested at a particular concentration) was done at least twice and accompanied with an independent control (plant growing in water under the same conditions).

Primers 5′-GAGAGCGAGAACGAAGTGGGCTT and 5′-AATTGATTTTTTGTAGGCATCCCTC complementary to a 451-bp fragment of the wheat mitochondrial gene *Nad3-rps12* were kindly provided by Dr. H. Wintz (Plant Molecular Biology Institute, Strasbourg, France).

To study synthesis of DNA including H-mtDNA in coleoptiles, the seedlings (100) were cut and incubated in 5 ml solution of the labeled DNA precursor [^{3}H-methyl]thymidine (50 µCi/ml, Izotop, Russia) for 2 h at room temperature.

To isolate DNA, the coleoptiles or leaves of the etiolated wheat seedlings 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. Solid CsCl was added to the solution to achieve density 1.700 g/cm^3, and the mixture was centrifuged for 24 h in VTi-65.1 rotor at 50,000 rpm in an L8-70M ultracentrifuge (Beckman, USA) at 20°C. Then 0.2-ml fractions were collected from the tube bottom, and their density and the UV absorption value at 260 nm were measured. DNA from these fractions was precipitated with ethanol and dissolved then in 50 mM Tris-HCl buffer, pH 7.5, containing 25 mM EDTA. The DNA samples obtained were 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 and purified 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.

To determine DNA content, coleoptiles from 10 seedlings were isolated and ground in a mortar with pestle in liquid nitrogen. DNA amount was measured spectrophotometrically by determination of the differential absorption value at 270 and 290 nm [15] in acid extracts obtained from plant material after RNA removal by a modification of the procedure of Schmidt and Thannhauser [16].

Electron microscopic investigation of sections obtained from various organs (coleoptile, root) of wheat seedlings was carried out as described earlier [17].

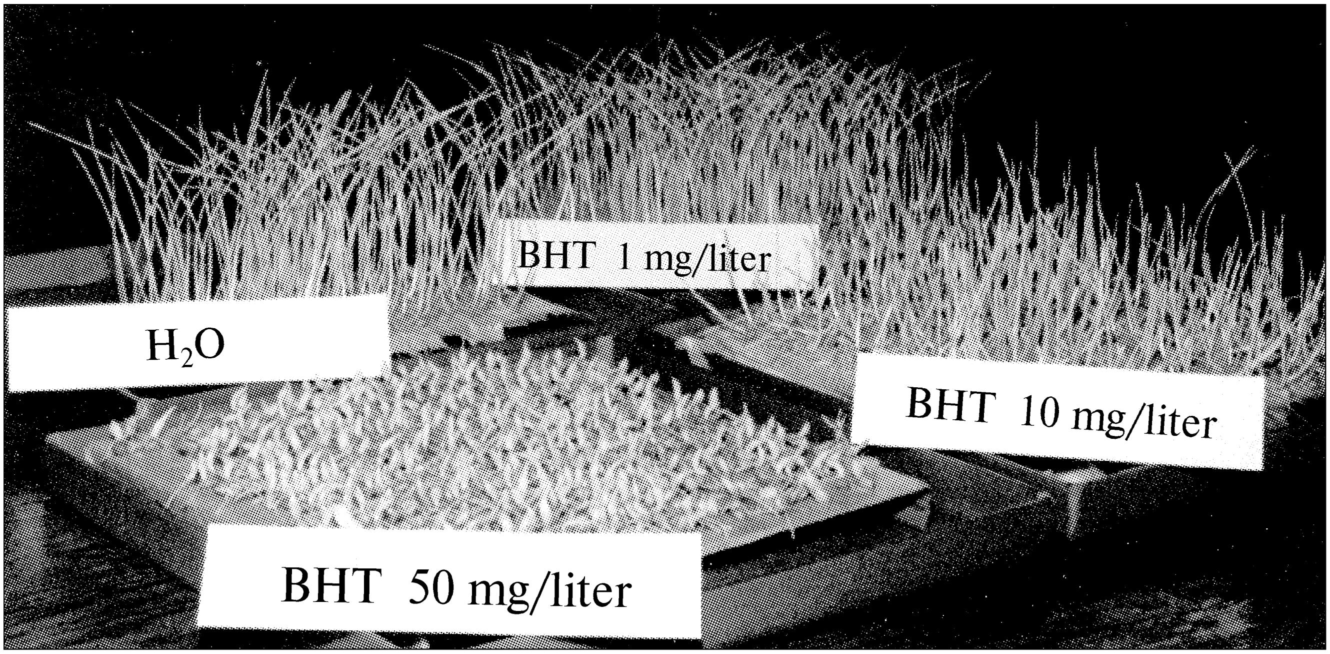


Fig. 1. Eight-day-old etiolated wheat seedlings. Numbers show BHT concentration (mg/liter) in the growth medium.

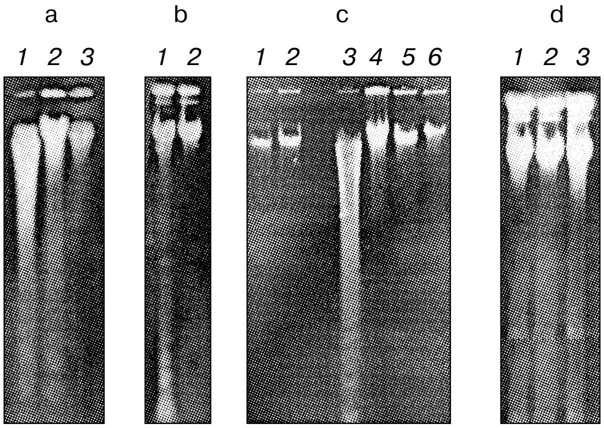


Fig. 2. Electrophoregrams of DNA isolated from coleoptiles (leaves) of etiolated wheat seedlings. a) DNA from coleoptiles of control seedlings of various age: *1*) 8-day-old seedlings; *2*) 6-day-old seedlings; *3*) 4-day-old seedlings. b) DNA from initial leaf of control 8-day-old seedlings: *1*) apical leaf part; *2*) basal leaf part. c) DNA from coleoptiles of: *1*) 4-day-old wheat seedlings grown in the presence of 50 mg/liter ($2.27\cdot10^{-4}$ M) BHT; *2*) 6-day-old seedlings grown in the presence of 50 mg/liter BHT; *3*) control 8-day-old seedlings grown in water; *4*) 8-day-old seedlings grown in the presence of 1 mg/liter BHT; *5*) 8-day-old seedlings grown in the presence of 10 mg/liter BHT; *6*) 8-day-old seedlings grown in the presence of 50 mg/liter BHT. d) DNA from coleoptiles of 8-day-old seedlings grown in the presence of: *1*) 50 mg/liter ($2.45\cdot10^{-4}$ M) 3,5-di-*tert*-butyltoluene; *2*) 50 mg/liter ($2.8\cdot10^{-4}$ M) sodium ascorbate, pH 5.5; *3*) control (seedlings grown in water under the same conditions).

RESULTS

Influence of BHT on seedling growth and morphology. We have chosen wheat seedlings as a model useful for investigation of the effect of BHT on intact plants. Standard methods of growing seedlings and their growth synchronization were developed already and the periodic synchronous cycles of DNA synthesis in coleoptile and leaf were observed and studied earlier [18]. Apoptosis with marked internucleosomal fragmentation of nuclear DNA [19, 20] and the appearance in the cells of specific vesicles containing active mitochondria producing heavy mitochondrial DNA were observed in the coleoptile of the etiolated 6-day-old wheat seedling [21-26].

We tested the influence of various BHT concentrations (1, 10, and 50 mg/liter) on the growth of etiolated wheat seedlings. Moderate growth retardation by BHT was clearly observed even at concentrations 1-10 mg/liter (Fig. 1). But at 50 mg/liter ($2.27\cdot10^{-4}$ M), the retardation of growth by BHT is expressed most strongly. Other xenobiotics (3,5-di-*tert*-butyltoluene, benzyl chloride) did not show a similar effect: wheat seedlings grown in the presence of these compounds at the same maximal concentration as for BHT did not differ morphologically from control plants [14]. Therefore, the observed plant growth regulating (inhibiting) effect of BHT seems to be due primarily to its antioxidant properties. It is interesting that in animals [27] a marked reversible physiological effect of BHT on activation and induction of various processes including

DNA methylation and others are most clearly seen at similar BHT concentrations (50 mg/kg body mass).

Influence of BHT on apoptotic DNA fragmentation. Apoptotic DNA fragmentation in the coleoptile cells of an intact control (grown in water) etiolated wheat seedling is a strongly programmed event in ontogenesis, and it appears clearly on the sixth day of the life of the seedling (Fig. 2a, lane *2*); in 8-day-old seedlings this process is strongly pronounced (Fig. 2a, lane *1*). Apoptotic DNA fragmentation was also detected in the initial leaf (Fig. 2b) of 8-day-old control seedlings. However, in the leaf this process was observed only in the apical part of the leaf blade (Fig. 2b, lane *1*), i.e., in the oldest leaf zone with non-dividing cells. This contrasts to situation in the coleoptile where most cells are synchronously involved in apoptosis. Thus, the aging of organs (initial leaf, coleoptile) in developing wheat seedlings, similarly to leaf aging in other plants [28], is accompanied by apoptosis. This corresponds to the idea that natural aging and death of plant leaves involves apoptosis [28, 29].

In contrast to control plants, in etiolated wheat seedlings grown in the presence of 1-50 mg/liter BHT apoptotic DNA fragmentation does not occur (Fig. 2c, lanes *4*-*6*). This means that BHT prevents apoptosis in the coleoptile cells. The inhibiting effect of BHT on apoptosis and seedling growth is quite specific, and it is most probably associated with the antioxidative properties of BHT. The non-antioxidant BHT analog 3,5-di-*tert*-butyltoluene lacking the hydroxyl group does not prevent the apoptotic DNA fragmentation at the same maximal concentration as used for BHT (Fig. 2d, lane *1*). In contrast to BHT, seedlings growing in the presence of ascorbic acid do escape from apoptosis in the coleoptile (Fig. 2d, lane *2*); ascorbic acid has no influence on the seedling growth and development even at relatively high concentrations (1 g/liter; $5.7 \cdot 10^{-3}$ M) [14]. It is most probable that, in contrast to BHT, ascorbic acid is not sufficiently hydrophobic to effectively penetrate into the plastid and mitochondrial membranes and to inactivate ROS formed in these organelles. Besides, it is well known that plants have a very high content of endogenous ascorbic acid and, therefore, the concentration of this agent used seems to be insignificant.

Influence of BHT on the content and synthesis of DNA in coleoptiles. DNA content in the coleoptiles of control seedlings increases for the first four days of the seedling life, and it reaches a maximum with the first maximum of the $O_2^{\bar{}}$ formation observed earlier [5]. After five days of seedling growth, the DNA content in the coleoptile starts to diminish, and it decreases about 2-fold in the 10-day-old seedling (Fig. 3), this seemingly being due to cessation of the nDNA synthesis [18] and apoptotic DNA fragmentation.

In contrast to control plants, the marked decrease with age in DNA content in coleoptiles did not occur during the entire observation period for a few days when seedlings were grown in the presence of BHT (Fig. 3). Thus, in the presence of BHT the decay of DNA is evidently postponed and the coleoptile lifespan should be increased by, at least, four days, i.e., by 30%.

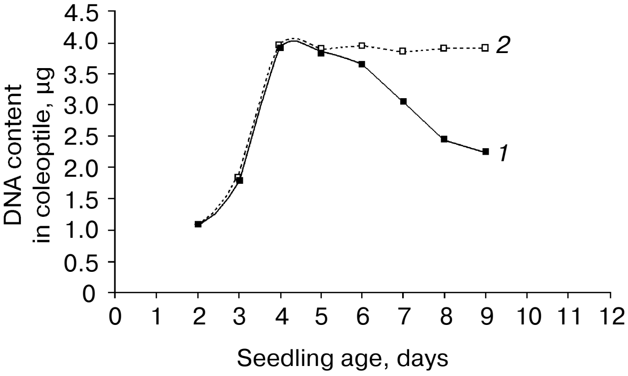


Fig. 3. DNA content in the coleoptile: *1*) control (grown in water) seedlings; *2*) seedlings grown in the presence of 50 mg/liter BHT.

In control seedlings, [^{3}H]thymidine is incorporated into coleoptile nuclear DNA (ρ = 1.700 g/cm^3) only until the fourth day of the life of the seedling [18]. After this period, [^{3}H]thymidine is incorporated into two DNA fractions (Fig. 4, a, c, e): H-mtDNA (ρ = 1.718 g/cm^3) and an unknown DNA with buoyant density ρ = 1.708-1.710 g/cm^3.

We were able to obtain a 451-bp fragment of the wheat mitochondrial gene *Nad3-rps12* by PCR in the presence of primers such as (5′)-GAGAGCGAGAACGAAGTGGGCTT and (5′)-AATTGATTTTTTGTAGGCATCCCTC when wheat H-mtDNA was used as a substrate. Along with other features [21-25], this shows that the H-mtDNA isolated by us is of mitochondrial nature. Formation of this fragment under the same condition during PCR with nDNA or unknown wheat DNA (ρ = 1.708-1.710 g/cm^3) was not observed (data not shown).

Unlike the control plants, in coleoptiles of seedlings grown in the presence of BHT the synthesis of heavy mitochondrial DNA (H-mtDNA) does not occur (Fig. 4, b, d, f). Only the unknown DNA with ρ = 1.708-1.710 g/cm^3 was synthesized in coleoptiles of the experimental plants after four days of the life of the seedling.

As shown earlier [21-24], synthesis of H-mtDNA is a specific feature of aging non-dividing plant cells: intensive H-mtDNA synthesis was observed in aging coleoptiles. This synthesis along with internucleosomal fragmentation of nuclear DNA and structural reorganization of the cytoplasm with the appearance of specific vacuolar vesicles [26] are markers of programmed cell death (apoptosis) in plants. BHT prevents the appearance of all these specific features of apoptosis.

BHT-induced changes in intracellular structures. Electron microscopic investigation of parenchyma tissues of the seedling coleoptiles and roots showed that the growing of seedlings in the presence of BHT (50 mg/liter)

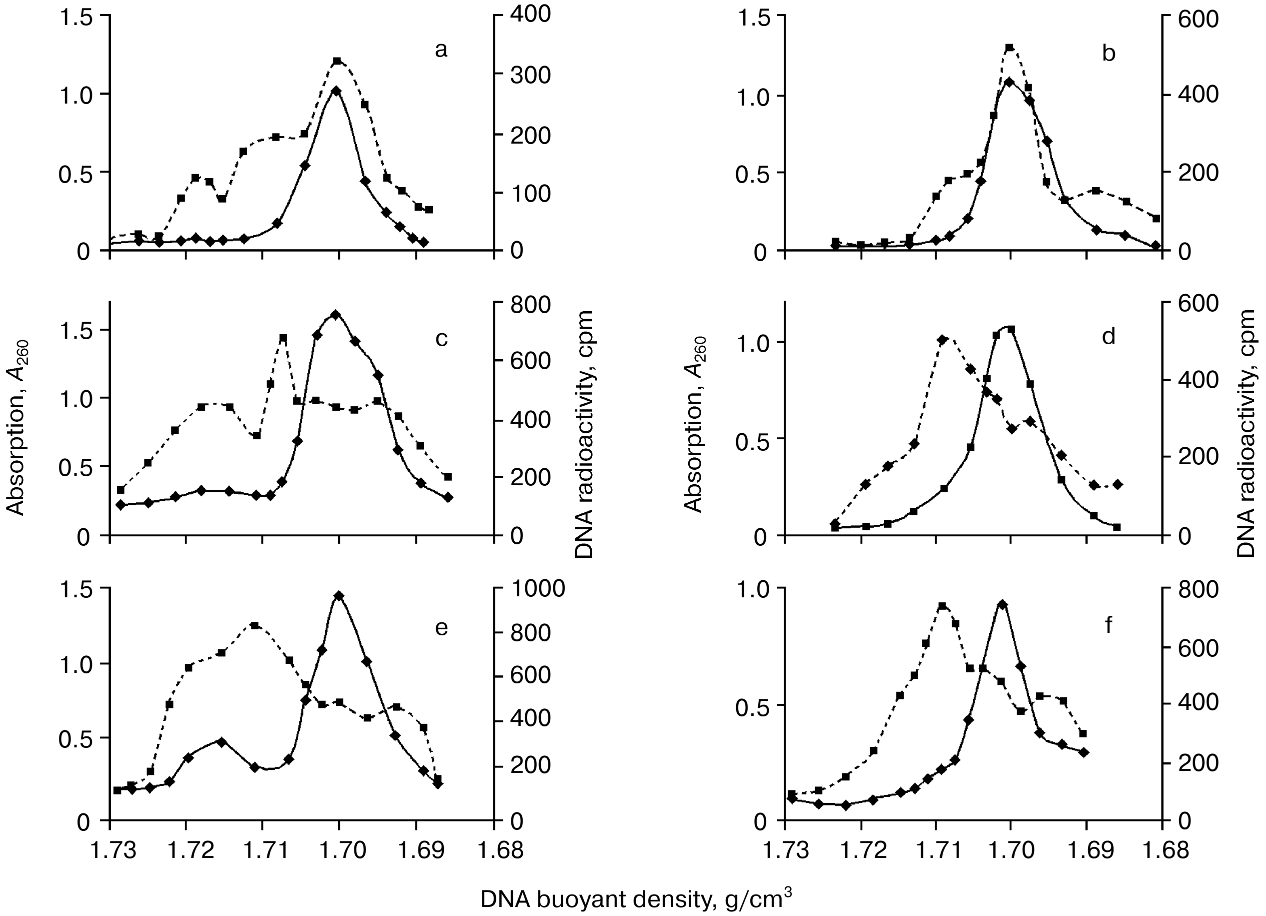


Fig. 4. Distribution in a CsCl density gradient of the radioactivity (dotted line) and UV absorption (solid line) of DNA isolated from wheat coleoptiles: a, c, e) control seedlings; b, d, f) seedlings grown in the presence of 50 mg/liter BHT; a, b) 4-day-old seedlings; c, d) 6-day-old seedlings; e, f) 8-day-old seedlings.

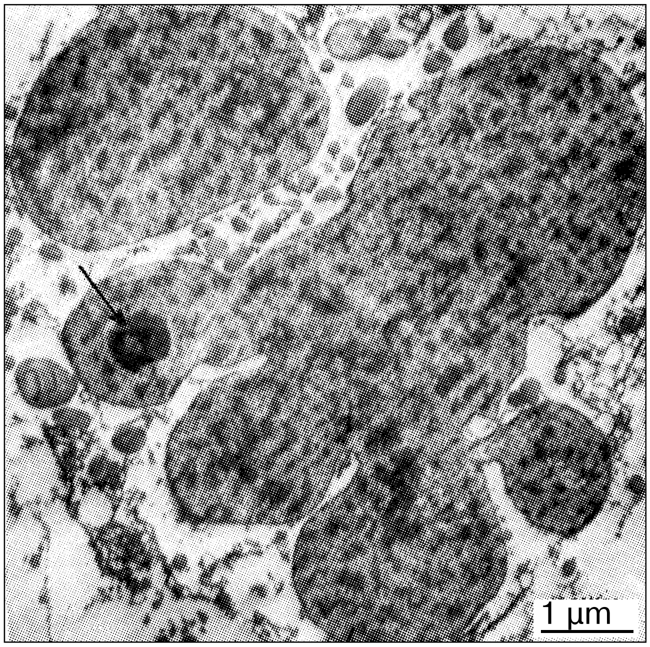
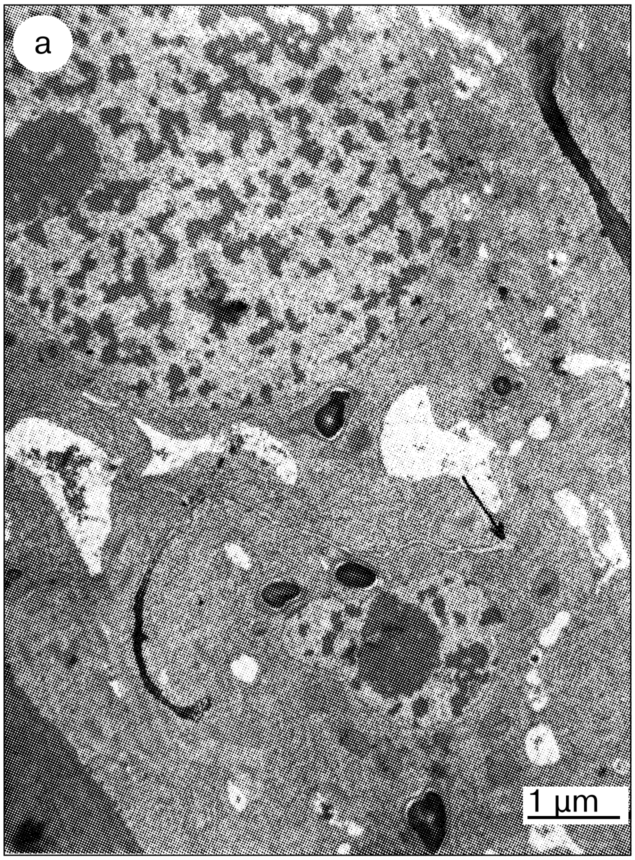


Fig. 5. Multi-bladed nucleus in a coleoptile cell of a 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. The arrow shows a nucleolus.

results in strong changes in all cellular organelles and the appearance of a few new and anomalous structures in the cell. In the coleoptile parenchymal cells of the 3-day-old control plants, the central area of the cell is usually occupied with vacuole, the oblate-shaped nucleus being reminiscent of a sort of lens, and it is usually attached to the cell wall. In the coleoptile cells of seedlings grown in the presence of BHT, but not in control plants, gigantic multi-bladed nuclei of irregular shape are present in the cytoplasm area protruding into the vacuole cavity (Fig. 5). These multi-bladed nuclei occupy about two thirds of the cell vacuole volume. Based on the ultrastructural parameters, these nuclei are polyploid.

Multinuclear cells are seen often in the roots of the 3- and 8-day-old wheat seedlings grown in the presence of BHT. The electron microscopic analysis in detail showed that this is due to incomplete cytokinesis that follows the division of the nucleus. Cell lamina and then the cell wall are only partially formed (Fig. 6). The cell wall divides the cell only up to two thirds or one half or sometime it is not even formed at all. Therefore, depending on the section plane, multinuclear cells can be observed. Multinuclear cells formed under the influence of BHT were observed



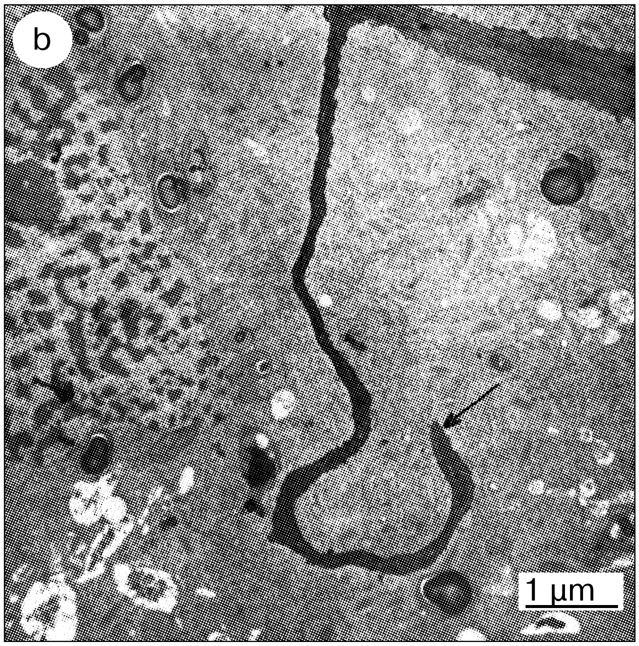


Fig. 6. Binuclear cell and defective cell wall observed in coleoptile of a 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT: a) binuclear cell; b) defective cell wall. Arrows show the cell walls formed incompletely.

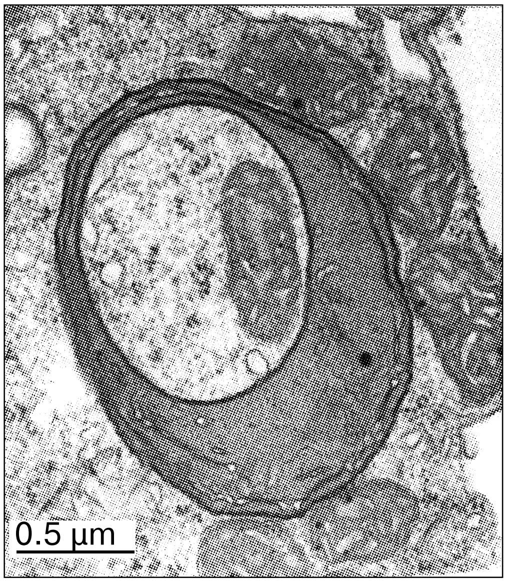


Fig. 7. An unusual circular plastid in a coleoptile cell of a 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. A mitochondrion is seen inside the plastid.

earlier in onion roots [13]. Thus, BHT does not inhibit the nuclear DNA synthesis (replication) (Fig. 3) but it suppresses the cell division. This means that the retarding effect of BHT on seedling growth (Fig. 1) may be due to inhibition of both cell elongation [5] and cell division.

In roots of etiolated wheat seedlings, BHT strongly induces the formation of pigments [14], probably carotenoids, which are known to be synthesized mainly in chromoplasts. Thus, we suggested that this antioxidant should significantly influence light-independent plastid differentiation in roots of etiolated plants, and, therefore, ROS may control both cell division and plastid differentiation [14].

Plastids that are seen in coleoptiles of the control etiolated seedlings are relatively non-differentiated plastids that may be related to the leucoplast group. The ultrastructure of plastids in wheat seedlings grown in the presence of BHT is drastically changed: the shape of the plastids becomes irregular and they most often have unusual closed circular structures. The membrane structure of these plastids is underdeveloped, the plastids do not contain or contain only a small number of thylakoids, and the main plastid volume is filled with matrix that often contains starch deposits (Fig. 7). In roots of seedlings grown in the presence of BHT, but not in control plants, large circular-form plastids are also present. The serial sections presented in Fig. 8 illustrate the ultrastructure of this type of plastids in the root of a 3-day-old seedling grown in the presence of BHT. It is worth mention that mitochondria are constantly seen inside such circular plastids. It is probable that under condition of decreased ROS content due to BHT [5] some specific and probably symbiotic relations between these functionally and morphologically different cellular organelles may originate. In any case, the overall plastid population formed in roots under the influence of BHT has a clearly unusual structure (Fig. 9); these plastids have doubled membranes and they contain two main components—a membrane system corresponding to an underdeveloped thylakoid system and the matrix. Along with membrane system mentioned, membrane vesicles fused with each other to form the prolamellar bodies can be seen (Fig. 9). In the control etiolated wheat seedlings, these organelles were not observed. From their ultrastructure, these organelles atypical for roots correspond to etioplasts (chloroplasts of etiolated plants), but they differ from etioplasts by their strongly elongated form with relatively small diameter; their length may reach up to 7 µm or more (Fig. 9). In contrast to control plants [26], in coleoptiles of the 8-day-old seedlings grown in the presence of BHT the formation in a cellular vacuole of specific vesicles containing mitochondria actively replicating mtDNA was not observed.

Under the influence of BHT, the mitochondria are less structurally changed compared with all other cellular organelles. Only the general morphology of mitochondria is changed in the coleoptile cells of the 3-day-old seedlings grown with BHT. These mitochondria are gigantic long organelles, but their internal ultrastructure is not changed, and it is similar to the mitochondrial structure in control plants. Marked changes in the mitochondrial ultrastructure induced by BHT were detected only in the root cells of the 8-day-old seedlings: these mitochondria are strongly diluted, their matrix being electron-transparent, and their crista are broken (data not shown).

Strong structural anomalies induced by BHT were detected in all organelles of the coleoptile parenchymal cells. For example, significant deviations from the well-known conception of the ultrastructure of the Golgi complex are revealed on investigation of this complex in the coleoptile cells of seedling grown with BHT. The dictyosome (the main component of the Golgi complex) forms in the middle part of the diluted chamber covered with membrane and filled with closed membrane particles. Parts of dictyosome cisterns remained open into this chamber (Fig. 10a). It is known that formation of the cell wall follows mitosis and depends on the activity of the Golgi complex. This complex participates in both synthesis and transportation of material that is needed for the formation of the cell wall. Besides, the Golgi complex takes part in formation of the plasma membrane. The defects in the cell wall formation and the distortion of the cell divisions observed might be due to changes in the structure of the Golgi complex induced by BHT.

We have observed that BHT also induces significant anomalies in the structure of the plasmatic reticulum of the coleoptile parenchymal cells in etiolated wheat seedlings. Specific closed double membrane structures formed around various cellular organelles by plasmatic membranes of the reticulum were detected in coleoptiles of 3-day-old etiolated wheat seedlings grown in the presence of BHT. A closed double membrane structure containing a few Golgi complexes is shown in Fig. 10b. A very unusual closed membrane structure with cytoplasm of

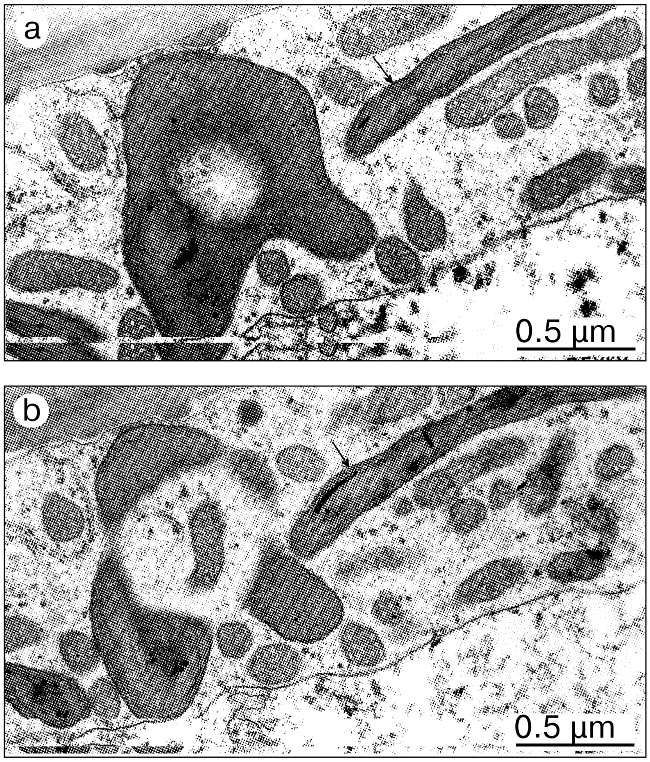


Fig. 8. Serial sections of a root cell of a 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. A circular plastid containing a mitochondrion inside and strongly elongated plastids atypical for roots are seen. These plastids are shown by arrows.

Fig. 9. Ultrastructure of the main plastid population formed in the roots of 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. Such plastids were not observed in roots of control plants. The membrane system (*1*) and a prolamellar body (*2*) are shown by arrows.

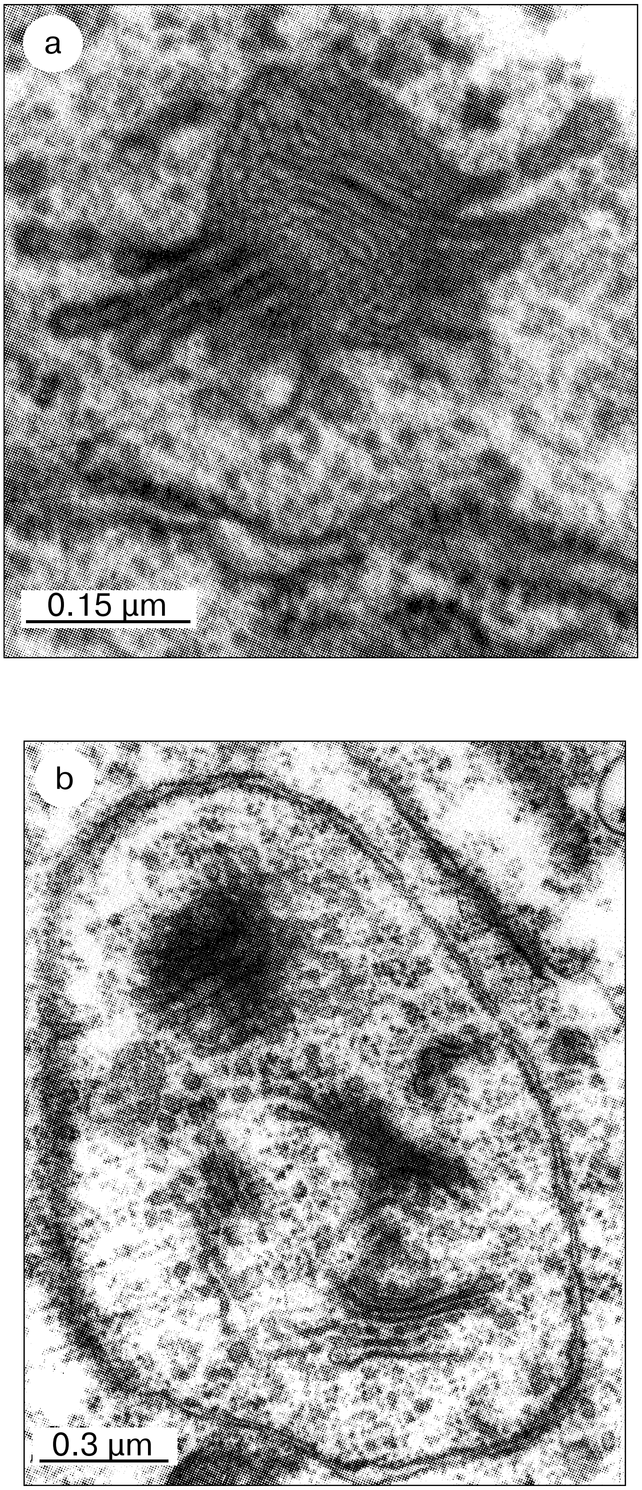


Fig. 10. Golgi apparatus and unusual cytoplasmic structure in a coleoptile of a 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. a) Ultrastructure of the Golgi complex changed under the influence of BHT; b) cytoplasmic structure formed by membranes of the endoplasmic reticulum under the influence of BHT. This unusual structure contains a gathering of dictyosomes.

Fig. 11. An unusual closed membrane structure in a coleoptile cell of 3-day-old etiolated wheat seedling grown in the presence of 50 mg/liter BHT. This structure is formed by membranes of the rough and smooth endoplasmic reticulum; it has heightened cytoplasm density and contains a mitochondrion.

different density and a mitochondrion located in the center is seen in Fig. 11. Chamber continuity of this closed membrane body is organized by membranes of the rough and smooth plasmatic reticulum. Thus, plasmatic reticulum can be considered as a relatively labile membrane system that can be changed and differentiated in correspondence with its functions. The antioxidant strongly affects the organization of this cellular structure, and ROS controlled by the antioxidant seem to participate in the differentiation of the cellular membrane structures (Golgi apparatus and others).

DISCUSSION

Our data show that BHT significantly affects plant growth and development. BHT is quite comparable with many phytohormones in the concentration effectiveness of its physiological influence on plants. This antioxidant has a marked retardant action and it seems to have a beneficial effect on the plants under conditions of oxygen stress. Together with other data [1-5], this shows that synthetic and natural antioxidants may be considered as a specific class of plant growth regulators. Unfortunately, the functional role of synthetic and endogenous antioxidants in plant development is still underestimated.

Coleoptile growth stops in 6-day-old control seedlings, and then the coleoptile length decreases [14]; this is associated with coleoptile aging that coincides with intense apoptotic internucleosomal fragmentation of nuclear DNA [19, 20] and the appearance in the cytoplasm of specific vesicles containing mitochondria intensively producing heavy mitochondrial DNA [21-26]. In contrast to control seedlings, in seedlings grown in the presence of BHT the coleoptile growth did not stop during the entire incubation period (11 days) [14]. This means that BHT inhibits the aging of the coleoptile and postpones its programmed death by more than 30%. Besides, we have found that BHT inhibits the age-dependent decrease in the total DNA and protein contents, age-specifically increased mtDNA synthesis, and prevents the apoptotic fragmentation of nDNA in the coleoptile. Thus, similarly to animals [8-11], BHT is an effective geroprotector in plants.

Synchronous growth and development of etiolated wheat seedlings [18] is accompanied with periodic and cyclic formation of superoxide ($O_2^{\bar{}}$) in the coleoptile and the initial leaf [5]. The first maximum in $O_2^{\bar{}}$ formation was observed on the fourth day of the seedling life [5], and it coincides with the cessation of nuclear DNA synthesis (Fig. 3); the second maximum of $O_2^{\bar{}}$ formation was detected on the seventh or eighth day of the seedling life [5] during a period of an intense apoptotic DNA fragmentation (Fig. 2a). We assumed that some critical level of $O_2^{\bar{}}$ formation in the cell or a combination of these levels may play a unique role of signals that trigger apoptosis accompanied with apoptotic internucleosomal DNA fragmentation and reorganization of the cytoplasm and subcellular structures [5]. BHT strongly inhibits the $O_2^{\bar{}}$ formation in coleoptile [5]. It is known that along with the ROS inactivation, the antioxidant BHT prevents depolarization of mitochondria [30], and this may inhibit the ROS-induced translocation from mitochondria into the cytoplasm of apoptotic factors including cytochrome *c* [31]. In any case, efficient prevention of apoptosis by BHT observed correlating with BHT inhibition of ROS formation in wheat coleoptiles [5] shows that ROS may trigger apoptosis in plants.

As found earlier [20-26], the synthesis of specific heavy mitochondrial DNA (H-mtDNA) in coleoptiles of control wheat seedlings begins on the fourth day of the seedling life during the period of the first maximum of $O_2^{\bar{}}$ formation. H-mtDNA is enriched with GC base pairs ($\rho = 1.718$ g/cm^3) when compared with nuclear DNA ($\rho = 1.700$ g/cm^3) and it consists mainly of circular molecules of various contour lengths [21]; H-mtDNA does not contain 5-methylcytosine [21], but it has N^6-methyladenine residues [22, 23]. Synthesis of H-mtDNA strongly increases with seedling age, and the amount of this DNA may correspond to 10-15% of that of nuclear DNA [25]. The increase in the amount of mtDNA coincides with the apoptotic fragmentation of DNA in the nucleus [19, 20]. Synthesis of H-mtDNA proceeds in mitochondria located in specific vacuolar vesicles [26] that are formed during this period. A signal to increase the synthesis of H-mtDNA and a massive accumulation of H-mtDNA seems to appear on the sixth day of the seedling life [19-22]. Unfortunately, the nature of this signal is unknown. We assume that ROS formed in the cell could be a good candidate for such signals since during growing of seedling in the presence of BHT, i.e., under condition when ROS are inactivated, specific vacuolar vesicles containing mitochondria were not formed and the synthesis of H-mtDNA was not observed.

BHT induces large structural changes in the organization of all cellular organelles (nucleus, mitochondria, plastids, Golgi apparatus) and it distorts the division of nuclei and cells. This results in the appearance of polyploid nuclei and multinuclear cells. In roots of etiolated wheat seedlings, BHT induces formation and differentiation of plastids with the appearance of plastids that are not typical for roots in general and for roots of etiolated plants in particular. These unusual plastids newly formed in roots of the BHT-induced etiolated wheat seedlings have some properties typical for chromoplasts or they are in fact chromoplasts. It is known that induced differentiation of chloroplasts into chromoplasts is accompanied by intense synthesis and accumulation of such carotenoids as lycopene, rhodoxanthin, astaxanthin, capsanthin, capsorubin, and others [32] that are the effective antioxidants. Agents inducing the formation of ROS (menadione, *tert*-butylhydroperoxide, paraquat) or prooxidants (diamide, buthionine sulfoximine) or an inhibition of the catalase activity strongly increase the transcription of many genes responsible for synthesis of carotenoids [32]. In our experiments, the well-known antioxidant BHT induced very strong synthesis of pigments, probably carotenoids [14], and it behaves like the prooxidants mentioned above. On the other hand, it cannot be ruled out that under some conditions depending on the oxygen concentration in different tissues and cells, BHT might be acting as a prooxidant. We are now investigating this point experimentally. However, there is no doubt that BHT modulates ROS content in the plant cell, and ROS may be agents of positive or negative control for plastid differentiation and carotenoid synthesis.

Antioxidant action is not limited only to changes induced in all known cellular organelles. BHT inhibits the normal age-dependent formation of some structures (vesicles in the aging coleoptile) or, on the contrary, it induces the appearance of structures that are atypical for given cells or tissues (relatively well-formed plastids in the roots of etiolated seedlings). Moreover, BHT induces formation of new subcellular membrane structures (Fig. 11). Thus, BHT acts as a structure reorganizing and forming agent that induces strong reorganization of the nucleus and cytoplasm in the plant cell. These effects of BHT should certainly be accompanied by the induction and switching over of activities of many genes and, in particular, genes that control plant growth and cellular differentiation. All specific morphological, biochemical, and cytological features of the action of BHT on the entire plant or organs and cells described were well reproducible in many experiments with growth of the etiolated wheat seedlings in the presence of BHT under standard conditions.

The multiple actions of BHT on plants seems to be primarily due to its antioxidative rather than its xenobiotic properties, since BHT strongly diminishes the superoxide content in the etiolated wheat seedlings [5], while other xenobiotics used at the same concentrations are physiologically inert [5, 14]. Therefore, it can be concluded that ROS controlled by BHT trigger apoptosis and structural reorganization of the cytoplasm with formation of specific vacuolar vesicles and mtDNA synthesis in them. It seems that proper modulation of the ROS level by BHT in the cell is responsible for the changes and distortions of the organelle structure observed and the appearance of new subcellular structures. ROS effectively control apoptosis and mitosis. ROS as powerful secondary messengers also regulate the differentiation of plastids and the Golgi apparatus, and they control cellular differentiation and plant growth and development.

We greatly thank engineer V. V. Kruglyakov for technical assistance in electron microscopy, V. V. Ashapkin for performing PCR, and H. Wintz for kindly supplying us with primers.

This work was supported in part by the Russian Foundation for Basic Research (grant Nos. 99-04-48090, 00-15-97920, and 01-04-48606).

REFERENCES

1. Scandalios, J. G. (1993) *Plant Physiol.*, **101**, 7-12.
2. Jabs, T. (1999) *Biochem. Pharmacol.*, **57**, 231-245.
3. Reichheld, J. P., Vernoux, T., Lardon, F., van Montagu, M., and Inze, D. (1999) *Plant J.*, **17**, 647-656.
4. Lorget, F., Kamel, S., Mentaverri, R., Wattel, A., Naassila, M., Maamer, M., and Brazier, M. (2000) *Biochem. Biophys. Res. Commun.*, **268**, 899-903.
5. Shorning, B. Yu., Smirnova, E. G., Yaguzhinsky, L. S., and Vanyushin, B. F. (2000) *Biochemistry (Moscow)*, **65**, 1357-1361.
6. Leshem, Y. Y. (1988) *Free Radic. Biol. Med.*, **5**, 39-49.
7. Vanyushin, B. F. (2001) *Usp. Biol. Khim.*, **41**, 3-38.
8. Harman, D., and Piette, L. H. J. (1966) *J. Gerontol.*, **21**, 560-565.
9. Emanuel, N. M. (1976) *Q. Rev. Biophys.*, **9**, 283-308.
10. Sharma, S. P., and Wadhwa, R. (1983) *Mech. Ageing Dev.*, **23**, 67-71.
11. Lippman, R. D. (1981) *J. Gerontol.*, **36**, 550-557.
12. Alekperov, U. K., Abutalybov, M. G., and Bagirova, A. D. (1976) *Genetika*, **12**, 47-50.
13. Alekperov, U. K., Abutalybov, M. G., and Askerov, I. T. (1975) *Tsitologiya*, **17**, 101-103.
14. Shorning, B. Yu., Poleshchuk, S. V., Gorbatenko, I. Yu., and Vanyushin, B. F. (1999) *Izv. RAN. Ser. Biol.*, No. 1, 30-38.
15. Spirin, A. S. (1958) *Biokhimiya*, **23**, 656-661.
16. Kirnos, M. D., Aleksandrushkina, N. I., and Vanyushin, B. F. (1981) *Biokhimiya*, **46**, 1458-1474.
17. Kirnos, M. D., Bakeeva, L. E., Volkova, S. A., Ganicheva, N. I., and Vanyushin, B. F. (1983) *Biokhimiya*, **48**, 1505-1512.
18. Kirnos, M. D., Volkova, S. A., Ganicheva, N. I., Kudryashova, I. B., and Vanyushin, B. F. (1983) *Biokhimiya*, **48**, 1587-1595.
19. Kirnos, M. D., Aleksandrushkina, N. I., and Vanyushin, B. F. (1997) *Biochemistry (Moscow)*, **62**, 1008-1014 (Russ.).
20. Kirnos, M. D., Aleksandrushkina, N. I., Shorning, B. Yu., Kudryashova, I. B., and Vanyushin, B. F. (1999) *Fiziol. Rast.*, **46**, 48-57.
21. Kirnos, M. D., Alexandrushkina, N. I., Zagorskaya, G. Ya., Kireev, I. I., and Vanyushin, B. F. (1992) *FEBS Lett.*, **298**, 109-112.
22. Kirnos, M. D., Aleksandrushkina, N. I., Shorning, B. Yu., Bubenshchikova, S. N., and Vanyushin, B. F. (1997) *Biochemistry (Moscow)*, **62**, 1587-1597 (Russ.).
23. Kirnos, M. D., Aleksandrushkina, N. I., Goremykin, V. V., Kudryashova, I. B., and Vanyushin, B. F. (1992) *Biokhimiya*, **57**, 1566-1573.
24. Vanyushin, B. F., Alexandrushkina, N. I., and Kirnos, M. D. (1988) *FEBS Lett.*, **233**, 397-399.
25. Kirnos, M. D., Aleksandrushkina, N. I., Bakeeva, L. E., Kazimirchyuk, S. B., Shorning, B. Yu., Alekseeva, V. A., Yaguzhinsky, L. S., and Vanyushin, B. F. (1999) *Biochemistry (Moscow)*, **64**, 307-317.
26. Bakeeva, L. E., Kirnos, M. D., Aleksandrushkina, N. I., Kazimirchyuk, S. B., Shorning, B. Yu., Zamyatnina, V. A., Yaguzhinsky, L. S., and Vanyushin, B. F. (1999) *FEBS Lett.*, **457**, 122-125.
27. Romanenko, E. B., Palmina, N. P., and Vanyushin, B. F. (1979) *Biokhimiya*, **44**, 1754-1761.
28. Yen, C. H., and Yang, C. H. (1998) *Plant Cell Physiol.*, **39**, 922-927.
29. Nooden, L. D., Guiamet, J. J., and John, I. (1997) *Physiologia Plantarum*, **101**, 746-753.
30. Novgorodov, S. A., Gudz, T. I., Mor, Yu. E., Goncharenko, E. N., and Yaguzhinsky, L. S. (1989) *Biokhimiya*, **54**, 206-212.
31. Balk, J., Leaver, C. J., and McCabe, P. F. (1999) *FEBS Lett.*, **463**, 151-154.
32. Bouvier, F., Backhaus, R. A., and Camara, B. (1998) *J. Biol. Chem.*, **273**, 30651-30659.