Biochemistry (Moscow), Vol. 65, No. 12, 2000, pp. 1357-1361. Translated from Biokhimiya, Vol. 65, No. 12, 2000, pp. 1612-1617.

Necessity of Superoxide Production for Development of Etiolated Wheat Seedlings

B. Yu. Shorning[1], E. G. Smirnova[2], L. S. Yaguzhinsky[1]*, and B. F. Vanyushin[1,2]

[1]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; fax: (095) 939-3181; E-mail: vanyush@genebee.msu.su
[2]Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, Timiryazevskaya ul. 42, Moscow, 127550 Russia

Received February 14, 2000
Revision received June 26, 2000

Abstract—It was found that production of superoxide ($O_2^{\cdot}$) is crucial for normal morphogenesis of etiolated wheat seedlings in the early stages of plant development. The development of etiolated wheat seedlings was shown to be accompanied with cyclic changes in the rate of $O_2^{\cdot}$ production both in the entire intact seedling and in its separated organs (leaf, coleoptile). First increase in the rate of $O_2^{\cdot}$ production was clearly observed in the period from two to four days of seedling development, then the rate of $O_2^{\cdot}$ production decreased to the initial level, and then it increased again for two days to a new maximum. An increase in $O_2^{\cdot}$ production in the period of the first four days of seedling development correlates with an increase in DNA and protein contents in the coleoptile. The second peak of increased rate of $O_2^{\cdot}$ production observed on the sixth or seventh day of seedling development coincides with a decrease in DNA and protein contents and apoptotic internucleosomal nuclear DNA fragmentation in the coleoptile. Incubation of seedlings in the presence of the antioxidant BHT (ionol) strongly affects their development but it does not influence the increase in DNA and protein contents for the initial four days of seedling life, and it slows down the subsequent age-dependent decrease in protein content and fully prevents the age-dependent decrease in DNA content in the coleoptile. A decrease in the $O_2^{\cdot}$ amount induced by BHT distorts the seedling development. BHT retards seedling growth, presumably by suppression of cell elongation, and it increases the life span of the coleoptile. It seems that $O_2^{\cdot}$ controls plant growth by cell elongation at the early stages of seedling development but later $O_2^{\cdot}$ controls (induces) apoptotic DNA fragmentation and protein disintegration.

Key words: antioxidant, apoptosis, BHT, DNA fragmentation, DNA synthesis, ontogenesis, ROS, plant, protein synthesis, superoxide, wheat

Reactive oxygen species (ROS) at high concentrations damage biological macromolecules and intracellular structures. This can significantly influence vital functions of cells and organisms depending on the effectiveness of protective and reparative systems in plants. In contrast to the destructive action of higher doses of ROS, at relatively low concentration ROS play a role in the signaling that controls some of the most important biological processes, e.g., mitosis [1-3] and apoptosis [4]. In particular, $O_2^{\cdot}$ and H_2O_2 are key inducers of apoptosis in plants. For example, $O_2^{\cdot}$ production in *Arabidopsis thaliana* mutants with distorted negative control of apoptosis is strongly increased [5]. The formation of H_2O_2 also increases during pathogen-induced cell death due to a hypersensitive response of the plant [6]. Strong inhibition of programmed cell death at low partial pressure of oxygen is also significant evidence for the participation of ROS in induced apoptosis [7] since ROS formation under this condition is suppressed [8, 9]. Antioxidants and enzymes of the protective antioxidative cell system decrease the ROS level in the cell, and they can prevent apoptosis in animal cells [10], the respective mechanisms of ROS inactivation possibly being significantly modified or even switched off during apoptosis [11].

The coleoptile in cereal plants is an organ with a very limited life span. It covers the primary leaf and protects it during seed germination and plant growth through the soil, then it ages and dies early in the process of seedling development [12]. The divisions and aging of cells in the wheat coleoptile are quite synchronous [13], and, therefore, this particular organ is very useful for studying mas-

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

* To whom correspondence should be addressed.

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sive apoptosis in plants [12]. Investigation of the role of ROS in the processes of growth and death of the coleoptile is of special interest.

The main goal of this work was to study the possible role of $O_2^{\cdot}$ in wheat seedling growth and morphogenesis and to investigate the dynamics of superoxide radical formation in etiolated wheat seedlings grown without or in the presence of the synthetic antioxidant BHT (ionol). Special attention was given to comparative analysis of the dynamics of the $O_2^{\cdot}$ production rate and age-dependent changes in DNA and protein contents in the coleoptiles of seedlings grown in the presence and absence of BHT.

MATERIALS AND METHODS

Seeds of Moskovskaya 39 variety of winter wheat (*Triticum aestivum* L.) were germinated for 24 h at 26°C on wet filter paper in a plastic cuvette; each sprouted seed was transferred into another 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; 50 mg/liter, $2.3 \cdot 10^{-4}$ M) or 10^{-4} M benzyl chloride or 3,5-di-*tert*-butyltoluene (Aldrich, USA), and the growth of the seedlings was continued in darkness at 26°C. To prepare BHT water solution, solid BHT was dissolved in ethanol and added to boiling water to the concentration used, then the weakly opalescent mixture was cooled to room temperature. An equivalent volume of ethanol was added to the water used for growing the control wheat seedlings.

The rate of $O_2^{\cdot}$ production in intact etiolated wheat seedlings of various age (from 1 to 11 days) and in their separate organs (leaf and coleoptile) isolated from 5- to 10-day-old seedlings was evaluated by differential spectra of reduced tetranitro blue tetrazolium chloride (the spectrum of a sample incubated without superoxide dismutase minus the spectrum of a sample in the presence of 1 μg/ml superoxide dismutase added). Two wheat seedlings (or coleoptiles or leaves) were placed in each of two cuvettes with 4 ml of incubation medium and incubated at 25°C for 1 h. Results of experiments carried out in daylight and in darkness are similar. The rates of $O_2^{\cdot}$ production in 3-day-old seedlings incubated for 1 h in daylight or in darkness were 1.58 and 1.55 μmol/h, respectively. In 7-day-old seedlings, the corresponding values were 1.42 and 1.46 μmol/h, respectively. The incubation medium contained 10 μM EDTA, 10 mM K_2HPO_4, pH 7.8, 1 mg/ml Triton X-100, and 0.05% tetranitro blue tetrazolium chloride [14]. The optical density of reduced tetranitro blue tetrazolium chloride in the incubation medium was measured spectrophotometrically at 530 nm using a Hitachi 557 spectrophotometer (Hitachi, Japan). According to [15], $\varepsilon = 15{,}000\ M^{-1} \cdot cm^{-1}$, and 2 μmoles $O_2^{\cdot}$ reduce 1 μmole of tetranitro blue tetrazolium chloride.

To determine DNA and protein contents, isolated coleoptiles were frozen under liquid nitrogen and ground with a mortar and pestle until fine powder was formed. The DNA content was measured by determining the difference in absorption values at 270 and 290 nm in acid extracts from the frozen plant material after removal of RNA by a modified Schmidt and Thanhauser procedure [16]. To determine protein content, the frozen ground plant material was suspended in lysing buffer solution containing 0.05 M Tris-HCl, pH 7.5, 0.025 M EDTA, and 1% sodium dodecyl sulfate and incubated at 25°C for 30 min. For more complete cell lysis, the procedure of freezing and thawing was repeated twice, then the lysate was centrifuged for 5 min at 12,000*g* at room temperature, and protein concentration was measured in the supernatant using Bradford's method [17].

RESULTS AND DISCUSSION

The high synchrony of wheat seedling development [13] make it possible to trace the rate of the $O_2^{\cdot}$ production during the process of plant development. We found that during the first eleven days of development of etiolated wheat seedling, the rate of $O_2^{\cdot}$ production measured by the appearance of $O_2^{\cdot}$ in the incubation medium during 1 h changes in a cyclical manner (Figs. 1 and 2). We observed three $O_2^{\cdot}$ production peaks that appear with 3-day period

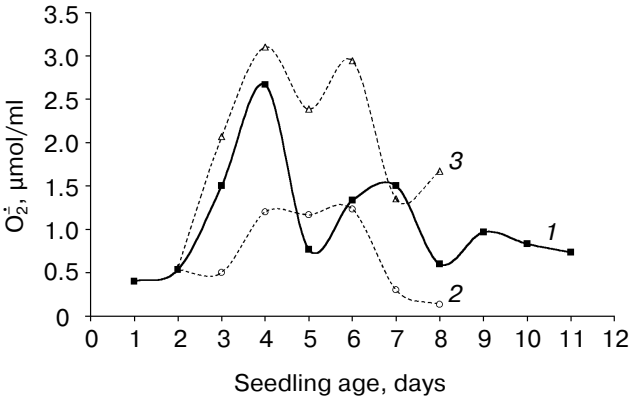

Fig. 1. Amount of $O_2^{\cdot}$ detected in the medium after incubation of intact etiolated wheat seedlings for 1 h in the presence of 1 mg/ml Triton X-100: *1*) control seedlings grown in water; *2*) seedlings grown in the presence of $2.3 \cdot 10^{-4}$ M BHT; *3*) seedlings grown in the presence of 10^{-4} M benzyl chloride. To evaluate amount of $O_2^{\cdot}$ evolved into the medium, the differential spectrum of reduced tetranitro blue tetrazolium chloride was registered in samples without or with addition of superoxide dismutase (1 μg/ml). Two wheat seedlings were placed in each of two cuvettes containing 4 ml of incubation medium and incubated at 25°C in daylight for 1 h. Absorption of reduced tetranitro blue tetrazolium chloride was measured at 530 nm. Abscissa, seedling age starting from seed imbibition (days); ordinate, amount of $O_2^{\cdot}$ evolved during 1 h.

during eleven days of the early ontogenesis of the plant. Maximal $O_2^{\cdot-}$ production was observed on the fourth day of seedling development, and then the amplitudes of the peaks decreased.

The dynamics of the increase in the rate of $O_2^{\cdot-}$ production (Fig. 1, curve *1*) and DNA (Fig. 3, curve *1*) and protein (Fig. 4, curve *1*) contents in coleoptile and the coleoptile length (data not shown) are practically the same up to the fourth day of seedling development. It is important to note that the maximal $O_2^{\cdot-}$ production occurs at the same time as the maximal DNA and total protein contents in the coleoptile. This shows that synthesis of DNA and protein is accompanied by $O_2^{\cdot-}$ production.

The increase in DNA content in the coleoptile ceases (Fig. 3) and the protein content starts to sharply decline on the fourth day of seedling development (Fig. 4), when the rate of $O_2^{\cdot-}$ production is maximal (Fig. 1). The rate of $O_2^{\cdot-}$ production in seedlings after the fourth day of development also declines strongly (Fig. 1).

The second period of increased rate of $O_2^{\cdot-}$ production begins on the fifth day, and it reaches its maximum on the sixth or seventh day of seedling development. At about the same time, the amount of DNA in the coleoptile begins to decrease (Fig. 3) and protein content continues to decline (Fig. 4). Apoptotic internucleosomal fragmentation of nuclear DNA in the coleoptile and the apical part of the primary leaf begins in this period [12].

It is known that apoptotic fragmentation of nuclear DNA proceeds trough at least two stages [18]. Long DNA fragments (50-100 kb) appear soon after the beginning of apoptosis [19], and the internucleosomal DNA fragmentation starts much later [18, 19]. We observed that the apoptotic internucleosomal DNA fragmentation appears during the second but not the first maximum of the $O_2^{\cdot-}$ production rate.

The second maximum of the $O_2^{\cdot-}$ production rate coincides with the beginning of superproduction of the heavy mitochondrial DNA (H-mtDNA) fraction [20] in special vacuolar vesicles containing active mitochondria [21]. On the eighth day of seedling development, the amount of H-mtDNA corresponds to 10-15% of the nuclear DNA. We suggested earlier that a signal for rapid synthesis and massive accumulation of H-mtDNA in the coleoptile appears during the fifth cycle of synchronous DNA replication in meristematic cells of the primary leaf (sixth day of seedling development) [20]. The nature of this signal is still unknown. We think that $O_2^{\cdot-}$, and especially some threshold level of $O_2^{\cdot-}$ production and content in the cell, may be this signal.

We determined the $O_2^{\cdot-}$ production rate in the separate organs (coleoptile and primary leaf) of etiolated wheat seedlings. Unfortunately, we were able to separate the leaf from the coleoptile without significant damage to each not earlier than on the fifth day of seedling development. Therefore, we studied the $O_2^{\cdot-}$ production rate in leaf and coleoptile of 5-day-old and older seedlings. It

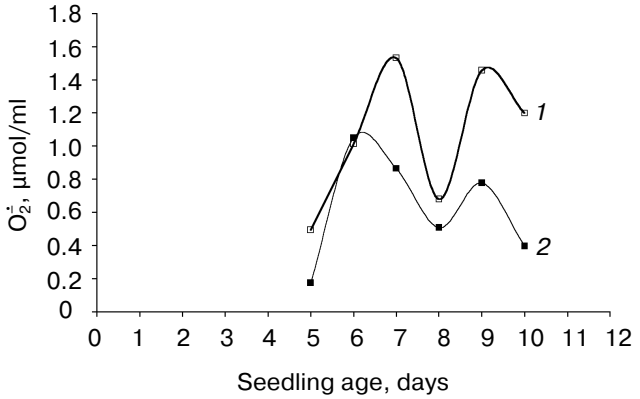

Fig. 2. Amount of $O_2^{\cdot-}$ detected in the medium after incubation of wheat seedling coleoptiles or leaves for 1 h: *1*) coleoptiles; *2*) leaves. Two leaves or coleoptiles were placed in each of two cuvettes with 4 ml of incubation medium and incubated at 25°C for 1 h.

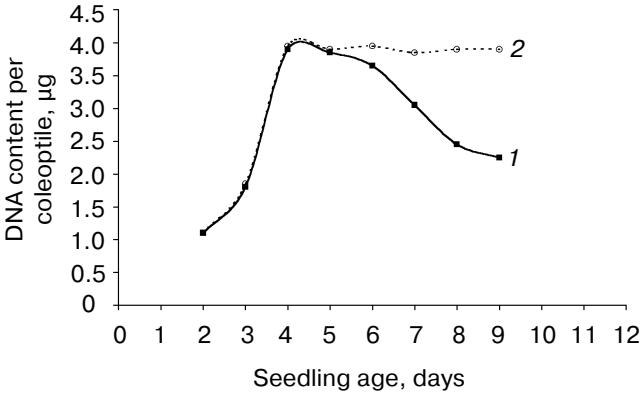

Fig. 3. DNA content in wheat coleoptiles: *1*) coleoptiles of control seedlings (grown in water); *2*) coleoptiles of seedlings grown in the presence of $2.3 \cdot 10^{-4}$ M BHT. The DNA content in μg per coleoptile is given.

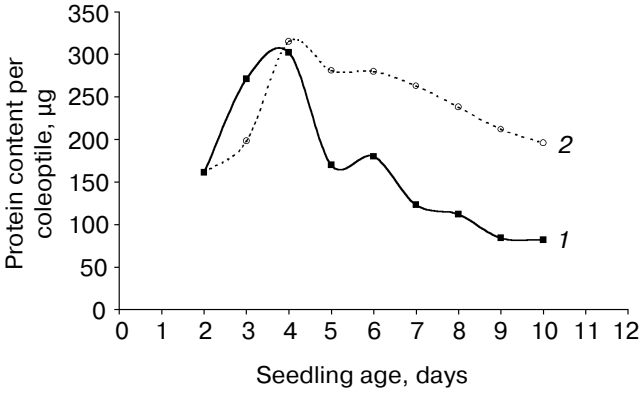

Fig. 4. Protein content in wheat coleoptile: *1*) coleoptiles of control seedlings (grown in water); *2*) coleoptiles of seedlings grown in the presence of $2.3 \cdot 10^{-4}$ M BHT. The protein content in μg per coleoptile is given.

was found that, like in the intact seedling (Fig. 1, second peak), the $O_2^{\cdot}$ production rate in the primary leaf and coleoptile (Fig. 2) increases in a period from five to six or seven days of seedling development, then it decreases to a minimum (eighth day) and then again increases to a maximum on the ninth day of seedling growth with subsequent decrease. Thus, the cyclic changes in the $O_2^{\cdot}$ production rate are observed both in leaf and coleoptile, these changes are similar, and they appear in the two plant organs rather synchronously (Fig. 2). But the amplitude of the changes in the $O_2^{\cdot}$ production rate in the coleoptile is significantly greater than in the leaf. This seems to be due to more expressed synchronous engagement of the majority of coleoptile cells in apoptosis and programmed organ death compared with that in the leaf. We clearly saw that, in contrast to the massive apoptosis in the coleoptile, apoptotic DNA fragmentation in the leaf of even an 8-day-old seedling does not occur in all cells; it is observed only in the cells of a relatively small and most senescent apical part of the leaf [12].

The regularities in changes in the $O_2^{\cdot}$ production rate in leaf and coleoptile are similar to that observed in intact seedling, and they reflect the general state of $O_2^{\cdot}$ production in a whole plant organism. The coincidence of activation periods of the $O_2^{\cdot}$ production in coleoptile and leaf shows that apoptosis is switched on in both these organs almost simultaneously.

The coincidence of maxima of the $O_2^{\cdot}$ production with the beginning of internucleosomal fragmentation of nuclear DNA, of decrease in protein content, and with synthesis of H-mtDNA suggested to us that we might be able to detect more directly the dependence of morphogenesis of a developing wheat seedling on $O_2^{\cdot}$ production by deliberate modulation of the $O_2^{\cdot}$ content in the cells with antioxidants. We used BHT as an antioxidant; it is well known as a ROS scavenger. BHT has a strong anti-aging effect and prevents apoptosis in animals [10, 22-25]. Besides, BHT, due to its structural similarity with ubiquinone, can inhibit oxygen consumption [26]. But BHT metabolites originating by P450-dependent oxidation may induce various pathological processes in animals [27].

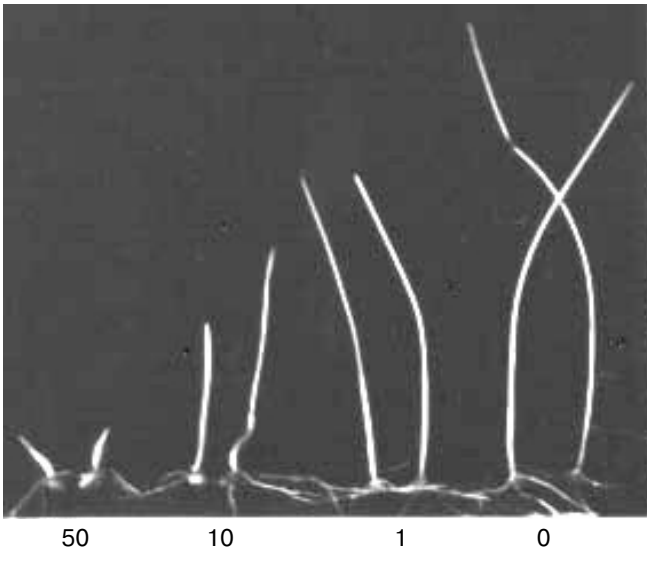

Fig. 5. Eight-day-old etiolated wheat seedlings. The numbers indicate the BHT concentration (mg/liter) in the medium.

We tested the effect of various BHT concentrations (1, 10, and 50 mg/liter) on the growth of etiolated wheat seedlings. At all concentrations used, BHT acts as a plant growth retardant (Fig. 5), the most strong retardant action of BHT being observed at 50 mg/liter in the medium ($2.3 \cdot 10^{-4}$ M) (Fig. 5). In contrast to control plants, the coleoptile in seedlings grown in the presence of BHT becomes much thicker, it does not dry, and its mass does not significantly decrease [28].

In etiolated seedlings grown in the presence of BHT (50 mg/liter), the yield of $O_2^{\cdot}$ is strongly (several-fold) suppressed and the periods of maximal $O_2^{\cdot}$ production rate are changed. To be sure that the observed effect of BHT is due mainly to its antioxidant but not ordinary xenobiotic properties, we analyzed the influence of xenobiotics devoid of antioxidant activity, such as benzyl chloride and the BHT analog 3,5-di-*tert*-butyltoluene, on the $O_2^{\cdot}$ production rate. Benzyl chloride, in contrast to BHT, significantly increases the $O_2^{\cdot}$ production rate in wheat seedlings (Fig. 1), but the BHT analog 3,5-di-*tert*-butyltoluene has no influence on it (data not shown). Benzyl chloride and 3,5-di-*tert*-butyltoluene do not affect the growth and development of wheat seedlings, whereas BHT inhibits growth of seedlings by about 80% without a large change in their mass [28]. Therefore, decrease in the $O_2^{\cdot}$ production rate, strong growth retardation, and other biological effects induced by BHT [28] are due mainly to its antioxidant properties. Thus, it is shown that seedling growth by elongation under conditions of significant inhibition of the $O_2^{\cdot}$ production rate by BHT (Fig. 1) is strongly suppressed (Fig. 5). This demonstrates that $O_2^{\cdot}$ controls the seedling growth by elongation.

DNA content in the coleoptiles of control plants starts to diminish during the second period of increase in the $O_2^{\cdot}$ production rate, when apoptotic processes are active. DNA content in 10-day-old seedlings decreases by about twofold (Fig. 3); this may be due mainly to apoptotic DNA degradation. In contrast, DNA content in coleoptiles of seedlings grown in the presence of BHT does not change in the period from four to at least ten days of seedling growth. Under these conditions, the age-dependent decrease in protein content in coleoptiles becomes slower (Fig. 4). This seems to be associated with inhibition of protein degradation, not with some BHT-dependent stimulation of protein synthesis. Unlike control plants, in coleoptiles of seedlings grown in the presence of BHT, vacuolar vesicles containing mitochondria are not observed and there is no H-mtDNA synthesis

(data not shown). Thus, $O_2^{\cdot}$ seems to induce also the formation of apoptotic vacuolar vesicles and the synthesis of H-mtDNA.

Because the wheat seedlings were grown in darkness, the production of $O_2^{\cdot}$ is not associated with photoinduced reactions but probably with mitochondria or NAD(P)H-oxidase of the cytoplasmic membrane.

Thus, $O_2^{\cdot}$ production in wheat seedlings is needed for their normal development. The periods of $O_2^{\cdot}$ production are related to the plant ontogenesis program. They induce, first, seedling growth by elongation and, second, apoptosis. The particular role of $O_2^{\cdot}$ may depend on the developmental stage of the seedling or organ: in the early stages of organ development, $O_2^{\cdot}$ seem to control the process of cell elongation but not DNA and protein synthesis; in the late developmental stages, $O_2^{\cdot}$ may participate in apoptosis.

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

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