Cyclical assessment of superoxide anion-radical generation and characterization of linking physiological parameters in wheat (*Triticum aestivum* L.) seedlings

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This study was conducted to evaluate the dynamics of superoxide anion radical ($O_2^{\bullet-}$) production rate and alterations in DNA content and permeability of cell membranes in etiolated wheat seedlings (*Triticum aestivum* L. cv. Harmony) as well as those grown under normal daylight regime. The results suggest that the development of etiolated wheat seedlings and those grown under normal daylight is accompanied by the periodic formation of $O_2^{\bullet-}$ which leads to alterations in DNA content as well as the permeability of cell membranes. The results also indicate that the first enhancement in the rate of $O_2^{\bullet-}$ generation was detected on the sixth and seventh days of development, after which the rate of $O_2^{\bullet-}$ production reduced in etiolated seedlings and those grown under normal daylight regime. The second maximum of the $O_2^{\bullet-}$ producing rate in developing and senescent organs occurred on the ninth day of plant development. The lowest values of all studied parameters, such as $O_2^{\bullet-}$ producing rate (37%), total genomic DNA concentration (58%) and electrolyte leakage (EL; 18%) were observed in etiolated wheat seedlings. Towards the end of the study period, DNA concentration and EL in organs of wheat seedlings declined, suggesting possible destruction of cellular organelles and the beginning of apoptotic processes. Overall, these results indicate that $O_2^{\bullet-}$ generation is decisive for normal morphogenesis, and it is an indispensable element of synchronous growth and development.

Keywords: DNA synthesis, membrane permeability, physiological parameters, superoxide radical, *Triticum aestivum* L.

MODERATE generation of reactive oxygen species (ROS) is necessary for normal physiology and successful adaptation to variable environmental conditions. However, their excessive production, for example, during severe environmental factors results in irreversible oxidative damage and dysfunction of cell components causing peroxidation of lipids, oxidation of proteins, damage of nucleic acids, enzyme inhibition and activation of programmed cell death (PCD) pathway[1]. To overcome ROS-induced oxidative degradation, plants have developed several cellular protective mechanisms and also stimulate the activation of antioxidative metabolism by increasing activities of enzymatic and non-enzymatic antioxidant compounds against post-stress oxidative stress.

To date, ROS are assigned a dual role – not only harmful, but also as stress signaling molecules controlling cellular processes such as growth, differentiation, development, stress response and cell death that attract particular attention of researchers[2,3].

This study was conducted to evaluate the dynamics of superoxide anion radical ($O_2^{\bullet-}$) producing rate and alterations in DNA content and permeability of cell membranes in etiolated wheat seedlings as well as those grown under normal daylight regime.

The experiments were carried out on etiolated wheat (*Triticum aestivum* L.) seedlings as well as those grown under normal daylight regime at different stages of development. The grains were germinated in a plastic pot containing moist filter paper at 26°C for 24 h. After germination, seedlings of equal length were transferred to polyethylene pots containing distilled water. The pots were then kept in a climate growth chamber (versatile environmental test chamber, Sanyo, Japan) at a constant temperature regime of 23° ± 1°C, relative humidity of approximately 75%, light intensity of 250 μmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h light/dark. The etiolated seedlings were grown in darkness at 23°C.

The rate of superoxide radical ($O_2^{\bullet-}$) production was measured as described by Shorning *et al.*[4], with slight modifications in intact coleoptiles and leaves of etiolated control wheat seedlings as well as grown under a normal daylight regime. This method is based on a spectrophotometric nitroblue tetrazolium (NBT, Sigma, USA) reduction assay. Plant material was placed in a test tube and incubated with or without 1 μl ml^{-1} superoxide dismutase (SOD; Sigma, USA) in darkness at 26°C in an incubation buffer (4 ml) containing 0.05% NBT. The reaction mixture contained 10 μl ethylenediaminetetraacetic acid (EDTA), 10 mM K_2HPO_4, 1 mg/ml Triton X-100 (Fluka, USA) and 0.05% NBT. After 1 h incubation in the dark, NBT reduction was measured spectrophotometrically (Cary 50 UV/Vis Scan, Varian, USA) at 530 nm.

Genomic DNA was isolated from the plant tissue accordingly to a protocol designed by Aljanabi and Martinez[5]. The frozen samples (100 mg) were thoroughly ground in a mortar with 400 μl of buffered sterile media (0.4 M NaCl, 10 mM Tris-HCl (pH 8.0) and 2 mM EDTA (pH 8.0)) with the addition of 40 μl 20% SDS and 8 μl 20 mg/ml proteinase K. Next, 300 μl of 6 M NaCl was added to the reaction mixture after incubation (65°C, 1 h) and stirred well using a Bio vortex V1 (Latvia) for 30 sec at maximum speed. After the homogenate was centrifuged at 14,000 *g* for 30 min, the supernatants were transferred to new tubes and then isopropanol was added, mixed well and incubated for 1 h (–20°C). The obtained DNA was

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Table 1. Total genomic DNA concentration (ng/µl) and purity (OD 260/280; OD 260/230) isolated from etiolated *Triticum aestivum* L. developing (first leaf) and senescent (coleoptile) organs*

Sample	NAC (ng/µl^{-1})	OD 260/280	OD 260/230	PL (mm)	Dilution
Leaf, 26°C, fourth day	817.04 ± 1.76	1.72	1.95	0.672	1.000
Leaf, 26°C, fifth day	746.33 ± 1.98	1.78	1.98	0.672	1.000
Leaf, 26°C, sixth day	832.05 ± 1.79	1.84	1.91	0.672	1.000
Leaf, 26°C, seventh day	573.21 ± 1.77	1.70	1.96	0.672	1.000
Leaf, 26°C, eighth day	437.33 ± 1.84	1.79	1.96	0.672	1.000
Leaf, 26°C, ninth day	586.67 ± 1.56	1.88	2.20	0.672	1.000
Leaf, 26°C, 10th day	548.35 ± 1.86	1.71	1.95	0.672	1.000
Leaf, 26°C, 11th day	358.06 ± 1.85	1.78	1.89	0.672	1.000
Coleoptile, 26°C, fourth day	79.02 ± 1.51	1.82	1.98	0.672	1.000
Coleoptile, 26°C, fifth day	57.61 ± 0.89	1.81	1.94	0.672	1.000
Coleoptile, 26°C, sixth day	46.19 ± 0.99	1.90	1.96	0.672	1.000
Coleoptile, 26°C, seventh day	40.03 ± 0.78	1.79	1.98	0.672	1.000
Coleoptile, 26°C, eighth day	45.32 ± 1.52	1.84	1.97	0.672	1.000
Coleoptile, 26°C, ninth day	34.31 ± 0.92	1.70	1.93	0.672	1.000
Coleoptile, 26°C, 10th day	24.21 ± 0.86	1.76	1.96	0.672	1.000
Coleoptile, 26°C, 11th day	21.88 ± 0.91	1.72	1.96	0.672	1.000

*NAC, Nucleic acid concentration (mean values ± SE calculated from four replicates (n = 4)); OD, Optical density; PL, Pathlength.

Table 2. Total genomic DNA concentration (ng/µl) and purity (OD 260/280; OD 260/230) isolated from *T. aestivum* L. developing (first leaf) and senescent (coleoptile) organs grown under normal daylight treatment

Sample	NAC (ng/µl^{-1})	OD 260/280	OD 260/230	PL (mm)	Dilution
Leaf, 26°C, fourth day	1025.33 ± 1.76	1.79	1.97	0.672	1.000
Leaf, 26°C, fifth day	872.04 ± 1.97	1.88	1.98	0.672	1.000
Leaf, 26°C, sixth day	781.33 ± 1.01	1.70	1.96	0.672	1.000
Leaf, 26°C, seventh day	1138.67 ± 1.67	1.81	1.95	0.672	1.000
Leaf, 26°C, eighth day	1047.33 ± 1.70	1.86	1.98	0.672	1.000
Leaf, 26°C, ninth day	982.10 ± 1.79	1.89	1.93	0.672	1.000
Leaf, 26°C, 10th day	506.81 ± 1.22	1.82	1.92	0.672	1.000
Leaf, 26°C, 11th day	580.12 ± 1.75	1.93	2.11	0.672	1.000
Coleoptile, 26°C, fourth day	193.71 ± 0.35	1.82	1.98	0.672	1.000
Coleoptile, 26°C, fifth day	169.49 ± 0.54	1.89	1.92	0.672	1.000
Coleoptile, 26°C, sixth day	175.39 ± 0.55	1.83	1.92	0.672	1.000
Coleoptile, 26°C, seventh day	183.78 ± 0.99	1.77	1.95	0.672	1.000
Coleoptile, 26°C, eighth day	153.39 ± 0.64	1.82	1.93	0.672	1.000
Coleoptile, 26°C, ninth day	147.68 ± 0.81	1.79	1.94	0.672	1.000
Coleoptile, 26°C, 10th day	137.02 ± 0.46	1.72	1.98	0.672	1.000
Coleoptile, 26°C, 11th day	126.82 ± 0.49	1.85	1.96	0.672	1.000

precipitated twice with 70% (v/v) ethanol, air-dried and dissolved in TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

DNA concentration in an aqueous phase (TE buffer) and its purity were assessed spectrophotometrically (Shimadzu BioSpec-nano, Japan) by the examination of absorption ratios (ARs) A_{260}/A_{280} and A_{260}/A_{230} using 1 µl biological specimen[6]. For all isolated biological specimens, the A_{260}/A_{280} ratio was between 1.7 and 1.9 (Tables 1 and 2). The absorbance scans demonstrated symmetrical peaks at A_{260}, indicating that the genomic DNA from leaves and coleoptiles showed high purity with the absence of proteins and phenolic compounds.

The permeability of cell membranes was measured by recording the electrical conductivity, as described previously[7]. Leaf and coleoptile samples were rinsed with deionized water and taken in test tubes containing 15 ml of double-distilled water and incubated in darkness for 24 h at room temperature (25°C; to prevent the loss of electrolytes induced by the light). Measurements of conductivity of the solution (initial conductivity) were done after 24 h using a conductivity meter (Mettler Toledo 980-K19/120 Conductivity cell, Schwerzenbach, Switzerland). Next, the tubes were then kept at 100°C in a boiling water bath with deionized water for 15 min, cooled down to room temperature, and their respective electric conductivity C_t values were re-measured (final conductivity) by a conductivity meter.

All data obtained from the experiments were subjected to analysis of variance and analysed using the SPSS

software package, version 13.0. The results obtained were presented as mean ± SE ($n = 5$), and differences at $P \leq 0.05$ were considered statistically significant.

The results of this study demonstrate cyclic changes in the rate of $O_2^{\bullet-}$ generation in developing and senescent organs of etiolated wheat seedlings and those grown under normal daylight during the first 11 days of development. The results suggest that the first enhancement in the rate of $O_2^{\bullet-}$ generation was detected on the sixth and seventh days of development, after which the rate of $O_2^{\bullet-}$ production declined in etiolated seedlings and those grown under the normal daylight regime (Figures 1 and 2). The second maximum of the $O_2^{\bullet-}$ producing rate in developing and senescent organs occurred on the ninth day of plant development (Figures 1 and 2). In contrast to etiolated wheat seedlings, the $O_2^{\bullet-}$ producing rate substantially increased in green seedlings grown under normal daylight (Figures 1 and 2).

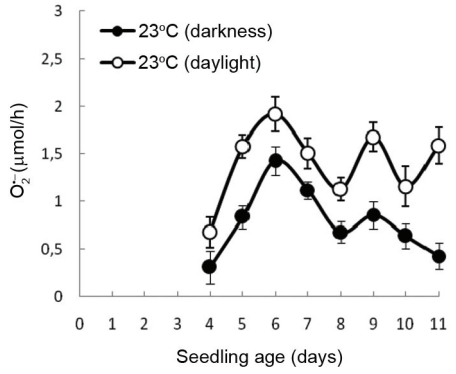

Figure 1. Superoxide anion radical ($O_2^{\bullet-}$) producing rate in etiolated developing organs of wheat seedlings and those grown under normal daylight conditions at different developmental stages. Data are presented as means ± SE ($n = 5$).

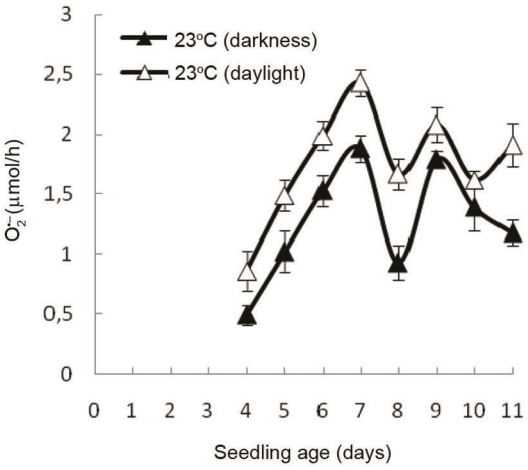

Figure 2. Superoxide anion radical ($O_2^{\bullet-}$) producing rate in etiolated senescent organs of wheat seedlings and those grown under normal daylight conditions at different developmental stages. Data are presented as means ± SE ($n = 5$).

The present study demonstrates that the first maximum of $O_2^{\bullet-}$ coincides with the highest increase in DNA content (832 ng/μl) in etiolated first leaves (Table 1). Unlike etiolated seedlings, in the initial leaves grown under normal daylight regime, DNA concentration reduced by 15% in five-day-old wheat seedlings (Table 2). However, in six-day-old wheat seedlings, DNA concentration reduced by 24% (Table 2). In the current context, although the $O_2^{\bullet-}$ producing rate peaked during the late developmental time (ninth day) in the initial leaves grown under normal control conditions, DNA content was not significantly reduced at this period (Table 2). On the other hand, DNA content had gradually elevated (43%) in etiolated nine-day-old leaves (Table 1). Unlike the initial leaves, in etiolated coleoptiles which function comparatively for a short period[8,9], DNA content decreased on the fourth day of the development and continued to decrease (49%) when the first maximum of the rate of $O_2^{\bullet-}$ was maximal (Table 1). In the present study, the secondary peak of the $O_2^{\bullet-}$ producing rate on the ninth day of the developmental stage matches with a reduction in DNA amount (57%) in etiolated coleoptiles compared to the early developmental stages (fourth day) (Table 1). However, although the $O_2^{\bullet-}$ producing rate reached a peak level in coleoptiles grown under normal daylight on the seventh day of development, an enhanced level of DNA concentration (17,539 ng/μl) was detected on the sixth day of development (Table 2). Moreover, the highest level of DNA concentration was noted on the seventh day of development (18,378 ng/μl) (Table 2).

The results indicate pronounced cyclical alterations of the permeability of the plasma membrane in etiolated initial leaves and coleoptiles, and seedlings grown under normal light conditions (Figures 3 and 4). The experiments reveal that permeability of the plasma membrane is permanent in etiolated initial leaves in spite of the two electrolyte leakage (EL) peaks at the early developmental stages, whereas in coleoptiles it is gradually elevated at the late stages of ontogeny. Two EL peaks – on the fourth and ninth days of development in etiolated developing organs, and three EL peaks on the 4th, 10th and 12th days of development for seedlings grown under normal daylight (Figures 3 and 4) were observed. In contrast to initial leaves of developing wheat seedlings, in etiolated coleoptiles and seedlings grown in the presence of light four EL peaks were observed on the 4th, 7th, 10th and 12th days of development (Figures 3 and 4).

The results demonstrate cyclic alterations in the rate of $O_2^{\bullet-}$ generation in developing and senescent organs of etiolated wheat seedlings and those grown under normal daylight during the first 11 days of development. It is known that the production of $O_2^{\bullet-}$ in etiolated wheat seedlings is not associated with photoinduced reactions, but probably with mitochondria or NAD(P)H-oxidase of the cytoplasmic membrane[4]. The increase in the rate of $O_2^{\bullet-}$ generation in green wheat seedlings grown under normal

daylight is likely due to chloroplasts that are presumed to be one of the major cellular organelles in plants responsible for ROS production in lightness[10]. Moreover, the rate of $O_2^{\bullet-}$ generation for seedlings grown under normal daylight increases due to the decomposition of water[11]. It has been noted that the amplitude of changes in the $O_2^{\bullet-}$ production rate in senescent organs is significantly higher than in the developing organs. This effect might be related to the synchronous engagement of the majority of coleoptile cells in the PCD process compared with that in the primary leaf. The results are consistent with those of Shorning *et al.*[4] who also found cyclic changes in the rate of $O_2^{\bullet-}$ generation in intact seedlings and in isolated organs of *T. aestivum* L., namely on the sixth day of development in the first leaves and on the seventh day of development in coleoptiles. Moreover, they demonstrated that the first maximum of $O_2^{\bullet-}$ generation coincided with a reduction in DNA and protein contents and a cessation of DNA replication, but the second maximum was related to the beginning of apoptotic DNA fragmentation in the sensecent organs of wheat seedlings[4]. In addition, results

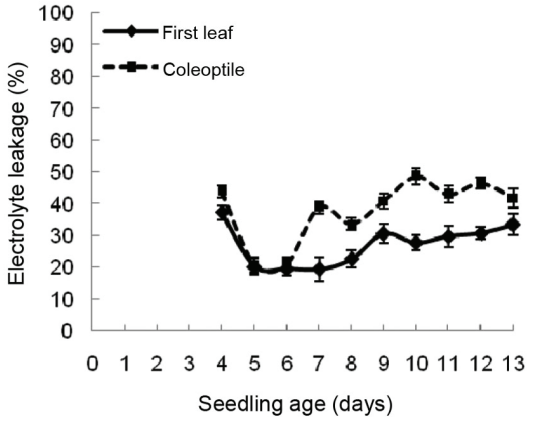

Figure 3. Electrolyte leakage in etiolated developing and senescent organs of wheat seedlings at different developmental stages. Data are presented as means ± SE (n = 5).

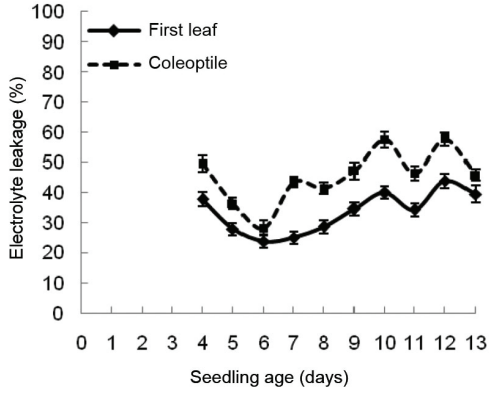

Figure 4. Electrolyte leakage in developing and senescent organs of wheat seedlings grown under normal daylight conditions at different developmental stages. Data are presented as means ± SE (n = 5).

from previous experiments also indicate the changes in $O_2^{\bullet-}$ producing rate during the study period in leaves peaking on the sixth day of development[12]. Based on the results described above, it has been suggested that periodic and cyclic production of $O_2^{\bullet-}$ accompanies the ontogenesis of wheat seedlings and it is a physiologically essential element of synchronous growth and development.

It was found that a cyclic formation of $O_2^{\bullet-}$ takes place in etiolated wheat seedlings. This process coincides with the periodical synchronous alterations of DNA synthesis in coleoptiles and the initial leaves. The reduction of DNA concentration in the initial leaves grown under normal daylight regime could be related to the destruction of cellular organelles and apoptotic processes. It is well established that PCD takes part in plant ontogeny, whose activation and progress are dependent on the intracellular redox conditions. As a confirmation of the assumption, earlier studies have highlighted that in *T. aestivum* L. growing during normal vegetation, the stimulation of PCD in initial leaf cells was already identified on the fifth day of development, whereas in etiolated leaves of control plants it was on the eighth day of development[8,11]. Moreover, in etiolated leaves, this process was developed only in the apical cap cells of the leaf blade, i.e. in the oldest zone of the leaf with non-dividing cells[11]. Apoptotic processes in the initial leaves grown under normal control conditions are assumed to have occurred earlier than those in etiolated seedlings. This suggests that the acceleration of PCD implies ROS generation due to photosynthesis in green leaves. DNA concentration was gradually elevated at the late stages of development. This effect was achieved by an active synchronous cell division of etiolated first leaves during this period. Recent findings support this observation. Kirnos *et al.*[13] detected five synchronous DNA replication cycles in etiolated control plants from the first to the seventh developmental stage. Previous studies have revealed that synthesis of nucleic acids was markedly elevated (10-fold) in green leaves of *S. oleracea* grown for 4 days under continuous artificial white light compared with the dark-grown leaves[14]. This reduction of DNA content in coleoptiles may suggest the starting of the apoptotic cell elimination process in *T. aestivum* L. Moreover, it reduces about 3.5-fold in the 11-day-old seedling (Table 1), this seemingly being triggered by the cessation of nDNA synthesis accompanied by most probable rapid DNA decay and apoptotic fragmentation in which endonucleases are known to be involved[15]. These observations are consistent with those obtained by other researchers. Smirnova *et al.*[16] determined that in the 14-day-old coleoptiles relative DNA content reduced by about 70% with age. Radha *et al.*[17] reported that the level of DNA degradation in maize seeds decreased with age. It was also shown that the concentration of DNA, as well as protein content in coleoptiles, reduced during the second period of the increase in the $O_2^{\bullet-}$ producing rate on the sixth and seventh days of

development[4]. It is well established that the growth of coleoptiles ceases in the six-day-old control seedlings accompanied by the intense internucleosomal fragmentation of nuclear DNA related to apoptotic processes[18] and persistent reorganization of the cytoplasm[19] in etiolated plants and growing during normal vegetation. Similar trends were found in seedlings grown in the presence of light where the amount of DNA in coleoptiles considerably reduced, this seemingly being triggered by the cessation of DNA synthesis and consequently, apoptotic death. When compared to the early stages of ontogenesis, in coleoptiles grown in the presence of light, DNA concentration considerably reduced at the late developmental stages. This finding might be connected with the latest developmental stages of coleoptile ontogenesis, when DNA synthesis stops and as a result, the amount of DNA in the coleoptile cells decreases. Consistent with this assumption, studies[20] have also suggested that after five days, the nuclear DNA concentration in coleoptiles of cereals begins to diminish[20]; it diminishes about two-fold in the ten-day-old seedlings. Wakiuchi *et al.*[21] reported that the level of DNA decreased rapidly on the fifth day of development in barley seedlings and then remained at the same level. Thus, the development and aging of coleoptiles are accompanied by PCD whose intensity is controlled by ROS, which in turn controls DNA synthesis.

The permeability of plasma membranes is considered an integral component of the functional characterization of plants that varies during ontogenesis. In this study, the enhancement in EL was more inherent in the initial leaves grown under normal light conditions, indicating perhaps strong thylakoid membrane injuries in leaf photosynthetic tissues. A relatively low intensity of generation of $O_2^{\bullet-}$ in the developing organs and enhanced EL at the late developmental stages can be described as the disruption of cell membrane structures, indicating alterations in the compound and structure of membrane integrity. Taking into account that coleoptiles function for a comparatively short life period in crop plants, an enhancement in EL in initial leaves of wheat seedlings at the late developmental stages might be connected with the senescence and an increase of oxidative stress, inducing destruction of permeability of plasma membrane. This presumption is also supported by the cyclic formation of $O_2^{\bullet-}$ in parallel with the EL level. It is well established that enzymes generating $O_2^{\bullet-}$, including the main ones, like NADPH oxidases, are membrane-bound and sensitive to alteration of the states of biomembranes[22]. This finding is consistent with the data obtained by previous studies. Takele[23] reported that the most dramatic alterations in the rate of EL were noticed during the late phase of post-flowering in maize and sorghum leaves. Rolny *et al.*[24] reported that EL enhanced during leaf senescence, which has been attributed to the disruption of cell membranes.

On the contrary, it was established that EL significantly altered in wheat coleoptiles during the development and senescing process. Very likely, this is associated with the generation of ROS leading to oxidative stress. In the present study, pronounced EL peaks in etiolated coleoptiles and seedlings grown in the presence of light at the late developmental stages might be associated with the irreversible membrane damage leading to PCD arising from increased ROS. Moreover, it is well established that an increase in EL is the main characteristic of senescence which represents the terminal stage of development. It is also characterized by degradation of macromolecules and remobilization of their constituents into other parts of plants[25]. On the other hand, a large increase in EL could be associated with an increased level of lipid peroxidation[26]. It is likely that in senescent cells, the proteins and lipids of membranes are degraded and this may lead to structural changes that cause loss of integrity and increased membrane permeability. This assumption is in consonance with earlier studies that demonstrated an increase of EL in senescing tissues[24,26]. In addition, our previous studies have reported an increase in the end-product of polyunsaturated fatty acid oxidation as well as an enhanced level of EL in senescent organs of wheat seedlings at various developmental stages during stress conditions[27,28].

Taken together, all of the data clearly show that the development of etiolated wheat seedlings and those grown under normal daylight regime are accompanied by the periodic formation of $O_2^{\bullet-}$, which leads to alterations in the genomic DNA content as well as the permeability of cell membranes. Furthermore, the highest $O_2^{\bullet-}$ producing rate was determined in green seedlings grown under normal daylight regime. In conclusion, these data clearly demonstrate that $O_2^{\bullet-}$ generation accompanies ontogenesis of wheat seedlings and it is a physiologically essential element of synchronous growth and development.

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Received 19 November 2020; revised accepted 24 November 2021

doi: 10.18520/cs/v122/i1/93-98

A balloon-borne experiment for quasi-Lagrangian frame of reference measurements of intrinsic frequency spectrum of gravity waves in the stratosphere

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In the present communication, first results from an experiment to measure intrinsic frequency spectrum of atmospheric gravity waves using balloon-borne quasi-Lagrangian frame of reference observations in the mid-stratosphere over a tropical station, Hyderabad (17.4°N, 78.2°E) are discussed. A zero-pressure polyethylene balloon with GPS-sonde payload was drifted at ~31 km altitude for a horizontal distance of ~100 km for measuring pressure, wind and temperature at 1 sec temporal resolution. The measured altitude of the balloon showed variability within ±100 m, thus ensuring a near horizontal drift. These observations are used to estimate the intrinsic frequency spectrum of gravity waves in the mid-stratosphere over an Indian observational site. The successful experiment has opened up a new avenue for studying not only the stratospheric gravity wave dynamics, but also for exploring the horizontal mapping of stratospheric trace gases.

Keywords: Balloon-borne experiment, gravity waves, intrinsic frequency spectrum, stratosphere, trace gases.

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