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Post-hypoxic Oxidative Stress in Aging Pea Seeds: II. Post-hypoxic Events

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ABSTRACT

A lot of air-dry seeds with lowered germination percentage can be subdivided on the basis of room temperature phosphorescence (RTP) into three fractions: I – strong seeds, II – weak seeds, and III – dead seeds. Using fraction II weak seeds separated from the seed lot according to RTP level, we have shown previously that seed imbibition is accompanied by the onset of oxygen deficiency, i.e., hypoxic conditions for the embryo. Oxygen deficiency originates from a higher respiration rate and greater limitation to oxygen diffusion through seed coats in fraction II seeds. Severe oxygen deficiency leads to seed death during the imbibition. The seeds with radicle protruded gave rise to morphologically defective seedlings, because of cell division disturbances in meristematic cells of embryo axis. Neither the high imbibition rate in fraction II seeds nor hypoxic conditions under seed coat impaired the DNA replication in the root tips of embryo axes. Both the cessation of DNA replication and DNA degradation took place only after radicle protrusion and aeration of imbibed fraction II seeds. Chemiluminescence measurements indicated an increase in the content of reactive oxygen species (ROS), mainly H_2O_2, generated by embryo axes. All observations were consistent with the idea that it is the post-hypoxic oxidative stress that caused the damage of cell division and appearance of abnormal seedlings from fraction II seeds. Various antioxidants decreased DNA degradation and increased seed lot germination capacity.

Keywords: antioxidants, chemiluminescence**,** DNA degradation, H_2O_2, porphyrin phosphorescence, room temperature phosphorescence
Abbreviations: aU, arbitrary units; **CL**, chemiluminescence; **ROS**, reactive oxygen species; **RTP**, room temperature phosphorescence

INTRODUCTION

The quality of seeds is known to decline during storage, and they become sensitive to various stresses, especially during their transition from quiescence to subsequent germination (Isely 1957). Appearance of seedlings with morphological defects is an early sign of seed aging. It is important to reveal what kind of stresses results in the formation of abnormal seedlings from aged seeds. For this purpose we applied the method of room temperature phosphorescence (RTP) to subdivide a lot of air-dry seeds into fractions comprising strong, weak (produced morphologically defective seedlings) and dead seeds (Veselova *et al*. 1999). Physiological and biochemical characteristics of fraction I strong seeds and fraction II weak seeds were compared with the aim to reveal the cause of abnormal seedling appearance.

The first part of this work has shown that germination of pea seeds in Petri dishes with their lower side submerged in water by 1-2 mm (direct contact with free water) gave rise to the imbibition stress resulting in the loss of viability. The number of dead seeds increased in comparison with their number in fraction III (Veselova and Veselovsky 2009).

The rate of water uptake during imbibition (without direct contact with free water) was higher in the fraction II seeds than in those of the fraction I. A higher respiration rate and greater limitation of oxygen diffusion by seed coats in fraction II seeds induced oxygen deficiency considered as hypoxic conditions for embryo - hypoxic stress. In order to assess the hypoxia level, we developed a non-intrusive luminescent method based on endogenous porphyrin phosphorescence of seeds. The results showed that hypoxia did not appear in fraction I seeds. In fraction II pea seeds, the hypoxia started to develop after 12- to 16-h imbibition. When oxygen deficiency enhanced, alive seeds did not germinate as they died due to suffocation during imbibition. At moderate level of oxygen deficiency (8-10 μM O_2/l or 20-80 arbirary units (aU) of porphyrin phosphorescence), the radicles protruded but the emerging seedlings exhibited various morphological defects (Veselova and Veselovsky, 2009).

The DNA replication in fraction II seeds started synchronously to that in fraction I seeds. The DNA replication during seed germination is known to occur after repairing the defects accumulated during storage of dry seeds (Osborne 1983). Hence, the air-dry seeds of fraction II did not contain non-repaired DNA lesions which might account for morphological defects in seedlings. Since hypoxia did not impair DNA replication (2C-4C) prior to radicle protrusion of fraction II seeds, it is evident that hypoxic conditions did not contribute directly to morphological abnormalities of seedlings.

Because the DNA replication in fraction II seeds ceased after the radicle protrusion, when oxygen availability for embryo was restored, it was proposed that the post-hypoxic oxidative stress in embryo axes was the only cause for DNA degradation and impairment of cell division, which resulted in appearance of morphologically abnormal seedlings. The aim of this work was to show that the appearance of seedlings with morphological defects is due to post-hypoxic oxidative stress.

MATERIALS AND METHODS

Seed material

Commercial pea (*Pisum sativum* L., cv. 'Nemchinovskii-85') seeds stored at room temperature (20-25°C) had germination percentage of 66%. Undamaged seeds of uniform size and weight 225 ± 25 mg were selected.

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Seed imbibition

Seed imbibition rate was measured by the weighing method.

In order to study antioxidant effects on imbibition rate, seeds were allowed to imbibe in Petri dishes (6 cm in diameter) between two layers of filter paper at 20°C. Four milliliters of tap water or antioxidant solution (propyl gallate [10^{-4} M] or carnasin [10^{-3} M]) were added into each dish of five replications containing 10 seeds. The increment in seed fresh weight was expressed as the percentage of initial seed weight. Each seed was weighed prior to and after 24 h imbibition. At this time, porphyrin phosphorescence of each seed was measured. After 48-h imbibition, seeds were transferred to rolled towels which were placed in an upright position for germination test.

Germination test

Non-fractionated seeds (five replicates of 10 seeds per treatment) were allowed to germinate at 20°C between filter paper Whatman No. 1 sheets moistened with tap water or antioxidant solution. Seeds were defined as germinated if they produced normal seedlings after 6-day germination. Seedlings with morphological defects and various growth disturbances were classified as abnormal according to ISTA Rules (ISTA 1996). Since the rate of oxygen uptake by dead seeds of fraction III was close to zero, porphyrin phosphorescence did not increase during imbibition of these seeds. The absence of porphyrin phosphorescence in seeds without radicle protrusion provides the mean to distinguish dead seeds from suffocated seeds having high level of porphyrin phosphorescence.

Room temperature phosphorescence in air-dry seeds and porphyrin phosphorescence in imbibing seeds and embryo axes

Both kind of phosphorescence were measured with a device described earlier (Veselova and Veselovsky 2009).

Chemiluminescence measurements

CL of embryo axes was measured in the presence of the chemiluminescent indicator luminol (5×10^{-5}M in 50 mM Tris-HCl buffer) using a single photon counting device produced at Biophysics Department of Moscow State University and described in more detail previously (Tarusov and Veselovsky 1978). The signal from the photomultiplier tube (PMT-85, Russia) sensitive to visible light was transduced to the amplifier and then to the recorder. The sample adapted to darkness was kept in the chamber at a fixed temperature.

Porphyrin phosphorescence and CL of embryo axes of pea seeds were detected in a quartz chambers (diameter 16 mm). Five embryo axes weighing ~7 mg each were placed into the chamber and covered with 1 ml of tap water (for porphyrin phosphorescence detection) or luminol solution (for CL measurements).

Aqueous solutions of catalase (20000 U/mg, Sigma) and antioxidants (propyl gallate [10^{-4} M] and β-mercaptoethylamine [10^{-3} M]) were used as quenchers of ROS-induced CL.

Estimation of DNA replication

DNA replication in the root tips was estimated by the method described previously (Veselova and Veselovsky 2009).

DNA degradation

The root tips of pea seedlings emerged from fraction I and fraction II seeds were sampled after various imbibition times: fraction I seeds imbibed for 48 h before radicle protrusion, whereas fraction II seeds imbibed for 42 h. Hypoxia in fraction II seeds was detected by means of porphyrin phosphorescence for the last 14-16 h of imbibition before radicle protrusion. After radicle protrusion seeds were aerated (incubated in air on filter paper moistened with water or with propylgallate (10^{-4} M).

DNA was measured in 5-mm root tips thoroughly ground with a mortar and pestle in liquid nitrogen. To determine the extent of DNA degradation as a function of post-hypoxic period, embryo axes were placed in liquid nitrogen immediately after the end of hypoxia, as well as following 15-min or 6-h aeration after the termination of hypoxia.

A lysing solution (50 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1% SDS) was added to the grinded sample, and the mixture was incubated for 30 min at room temperature. NaCl was added to 1 M concentration, and the reaction mixture was deproteinized by careful shaking with chloroform:isoamyl alcohol (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. The precipate was dissolved in 50 mM Tris-HCl buffer (pH 7.5), containing 25 mM EDTA. The DNA samples were treated with DNAase-free ribonuclease A (50 μg/ml) for 20 min at 37°C, and DNA was precipitated again with the addition of three volumes of 96% ethanol. Similar aliquots of isolated purified DNA preparations were electrophoretically separated 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. The extent of degradation of stained DNA was visually estimated.

Experimental design and statistics

Each experiment was repeated 3-5 times. **Fig. 1** and tables contain mean values and their standard errors. **Figs. 2** and **3** show representative kinetics from five experiments.

RESULTS AND DISCUSSION

DNA replication

As cell division in embryo axes of pea seeds commences few hours after radicle protrusion, DNA replication was examined at the stage of preparation of embryo axis cells for division. The extent of DNA replication is evident from the increase in 4C/2C ratio. The fraction III dead seeds showed no changes in 4C/2C ratio (**Fig. 1**). The 4C/2C ratio increased both in fraction I and fraction II seeds up to the period of radicle protrusion. Because fraction II seeds exhibited higher rates of water uptake, the ratio 4C/2C rose faster in seeds of this fraction. The radicle protrusion in fraction II seeds took place earlier, i.e., at 40-42 h of imbibition, and in fraction I seeds it occurred after 46-48 h of imbibition. After radicle protrusion the 4C/2C ratio did not grow in fraction II seeds, whereas this ratio continued to increase in fraction I seeds. The retardation of DNA replication occurred concurrently with or immediately after radicle protrusion. In order to determine whether this retardation in fraction II seeds was due to post-hypoxic oxidative stress,

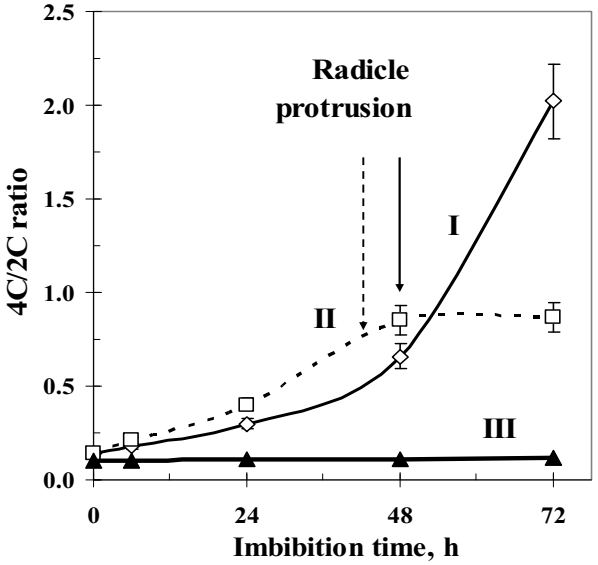

Fig. 1 Kinetics of 4C/2C ratio for nuclei in root tips from pea seeds of fractions I, II and III during imbibition. Dashed arrow indicates radicle protrusion in fraction II seeds, and solid arrow indicates that in fraction I seeds.

Table 1 Chemiluminescence of embryo axes from pea seeds (fraction I without hypoxia and fraction II after 14 h hypoxia), and effect of ROS inhibitors on chemiluminescence. Values indicate means ± standard errors (SE).

	Chemiluminescence, aU	Quenching of chemiluminescence, %		
		β-MEA	CAT	PG
Without hypoxia	32 ± 5	73 ± 5	78 ± 6	82 ± 6
Aeration after 14-h hypoxia	2450 ± 98	97 ± 3	95 ± 4	97 ± 4

CL and porphyrin phosphorescence of pea seed embryo axes were measured in parallel experiments.

Chemiluminescence (CL) as an indicator for generation of H_2O_2 in pea seed embryo axes

CL arises in the oxidation reaction of various phenolics with hydrogen peroxide (H_2O_2) catalyzed by peroxidase. The intensity of CL during peroxidase reaction is proportional to the amount of H_2O_2 as a most long-lived reactive oxygen species (ROS) (Tarusov and Veselovsky 1978; Veselovsky and Veselova 1990).

Roots of young seedlings and protruded embryo axes of germinating seeds emit weak CL, however the level of CL is manyfold enhanced in the presence of 10^{-5} M luminol. Injuring factors, such as heat shock, hypertonic solutions of salts or nonelectrolytes, toxic substances, etc., produce a significant increase in CL level (Tsoy *et al.* 1967; Pogosyan *et al.* 1978; Tarusov and Veselovsky 1978; Veselovsky and Veselova 1990). This phenomenon was used for assessment of stress-induced injury in plants.

CL needs the presence of oxygen. Under non-stressful conditions, the CL level is low and constant (32 ± 5 aU) over a long period.

When the chambers were closed hermetically, oxygen deficiency aroused inside the chamber because of embryo axes respiration. The appearance and subsequent rise in porphyrin phosphorescence provided evidence of increasing hypoxia.

The porphyrin phosphorescence level of 80 aU was attained at oxygen concentration in the chamber of 8-10 μM (Veselova and Veselovsky 2009). Similar levels of porphyrin phosphorescence were observed in imbibing seeds of fraction II. Such seeds were capable of protrusion but they produced abnormal seedlings with morphological defects (abnormal seedlings).

After 14-h exposure of embryo axes to oxygen deficiency in the hermetically closed volume (the level of porphyrin phosphorescence was 80 aU), the chambers were opened. During aeration of embryo axes (and respective contact with oxygen) the porphyrin phosphorescence quenched while CL increased (**Fig. 2**).

The quenching porphyrin phosphorescence proceeded in two phases: the initial rapid decline (within 5-10 s) was followed by a slow decrease that continued for several hours. The fast phase might reflect the interaction of oxygen with the cell surface, whereas the slow phase is related to oxygen diffusion into the tissues.

An increase in CL level also proceeded in two phases: the light emission increased within 5-10 s as much as 200 times, which was followed by a further slow increase up to the level of almost 2500 aU. This infers that hydrogen peroxide was generated both on the cell surface and inside the plant tissue.

An almost hundred-fold increase in CL level during the post-hypoxic period provides evidence for a sharp increase in H_2O_2 content in embryo axes. A similar CL increase was also detected upon intact root aeration during the post-hypoxic period (Vartapetian *et al*. 1974).

In the presence of catalase and antioxidants (propylgallate and β-mercaptoethylamine) the emission was quenched, confirming a large increase in total ROS forms (**Table 1**). Interestingly, the extent of CL quenching by catalase was higher during the post-hypoxic period, reaching 95-97%, as compared to 73-82% in the absence of hypoxia. This observation indicates that H_2O_2 is the main reactive oxygen species produced in the post-hypoxic period.

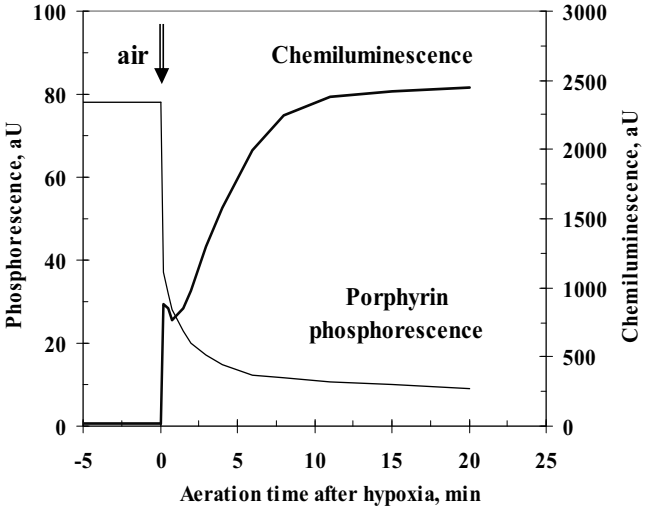

Fig. 2 Kinetics of porphyrin phosphorescence and chemiluminescence of embryo axes aerated immediately after the 14-h long hypoxia. Arrow indicates ambryo axes' contact with air.

Fig. 3 Electrophoregrams of total DNA isolated from embryo axes of pea seedlings. Lane 1 - fraction I seeds imbibed for 48 h without any hypoxia before radicle protrusion +6 h after radicle protrusion; lane 2 – fraction II seeds immediately after radicle protrusion; these seeds imbibed for 42 h to protrude(with hypoxia for the last 14 h of imbibition); lane 3 - fraction II seeds imbibed and protruded like in lane 2, but after the protrusion they were aerated for 6 h on filter paper moistened with water; lane 4 – fraction II seeds similar to lane 3, but aerated only 15 min on filter paper moistened with water; lane 5 – fraction II seeds similar to lane 3 but aerated for 6 h on filter paper moistened with propylgallate.

The CL level of embryo axes in imbibing seeds corresponds to the steady-state H_2O_2 concentration of about 1 μM (Veselovsky and Veselova 1990). Taking into account that the CL level in the post-hypoxic phase is enhanced by a factor of almost hundred, it appears reasonable to assume that H_2O_2 concentration increases by two orders of magnitude, up to 80-100 μM. Such high endogenous H_2O_2 concentration would be dangerous for living cells (Veselovsky and Veselova 1990; Khan and Wilson 1995; Wojtaszek 1997; Cheeseman 2007).

Various damages to plant cells were shown to increase H_2O_2 generation considerably, up to 10^{-5}-10^{-4} M (Abeles 1987).

DNA degradation

Fig. 3 shows the profile of DNA samples run in a gel. The DNA profile of the embryo axis from the control pea seeds without hypoxia had no signs of degradation (gel 1). Embryo axis that experienced 14-h-long natural hypoxia had similar profile when assessed immediately after hypoxia had ended (gel 2). However clear DNA degradation was observed in embryo axes which contacted with air for 6 h after hypoxia in Petri dish on wet filter paper (gel 4). In contrast, weak DNA degradation was observed when fraction II seeds experienced natural hypoxia being subjected to only 15-min aeration after hypoxia. This was evident from the weak DNA degradation (gel 3). This means that DNA degradation was developed during the post-hypoxic period.

Thus, post-hypoxic oxidative stress resulted in cessation of DNA replication as well as in degradation of existed DNA.

Post-hypoxic oxidative stress and antioxidants

Additional evidence that DNA degradation during the post-hypoxic period was caused by ROS (H_2O_2) generation is that this degradation was decelerated after incubation of embryos for 6 h in the presence of 100 μM propylgallate (**Fig. 3**, gel 5). In this case, the extent of DNA degradation was lower than in embryo axes incubated for 6 h on a filter paper moistened with water under natural aeration.

This result initiated the studying of the action of various antioxidants on seed water inflow and germination. **Fig. 4** shows the distributions of seeds by weight gain after imbibition for 24 h. The distribution of non-fractionated seeds in the presence of propylgallate and carnosine* demonstrates that antioxidants had little effect on the proportion of rapidly swelling imbibing fraction II seeds that usually produce morphologically defective seedlings. The peak in the fraction of low swelling seeds slightly shifted - from 60 to 70% of their weight gain.

However, seed germination tests have shown that the presence of antioxidants during the imbibition elevated the percentage of germinated seeds due to the decreased number of abnormal seedlings (**Table 2**). It means that normal seedlings could be produced from rapidly swelling seeds, and that detrimental influence of post-hypoxic oxidative stress was mitigated by antioxidants. The antioxidants low stimulated the rate of water uptake but reduced the amount of ROS generated during the post-hypoxic phase.

It is an important effect because without antioxidants any acceleration of imbibition resulted in the appearance of large number of abnormal seedlings.

CONCLUSION

A seed fraction producing abnormal seedlings with impaired cell division was fractionated from the lot of air-dry seeds.

Hypoxic conditions under the seed coat arise as a result of rapid imbibition of these seeds due to enhancement of oxygen uptake uncompensated by oxygen diffusion that follows from the appearance of porphyrin phosphorescence. Neither the imbibition damage at the first stage of the water uptake, nor hypoxic conditions suppressed the DNA replication in meristematic cells. The arrest of the DNA replication and degradation of existed DNA occurred only after the radicle protrusion and aeration of imbibed seeds of fraction II. The increase in the amount of ROS, mainly H_2O_2, generated by embryo axes is an evident cause of DNA damage. It is the post-hypoxic oxidative stress that caused the cell division disturbance and appearance of abnormal seedlings from fraction II seeds.

* carnosine – animal histidine-containing di peptide exhibiting antioxidant properties (Boldyrev 1993)

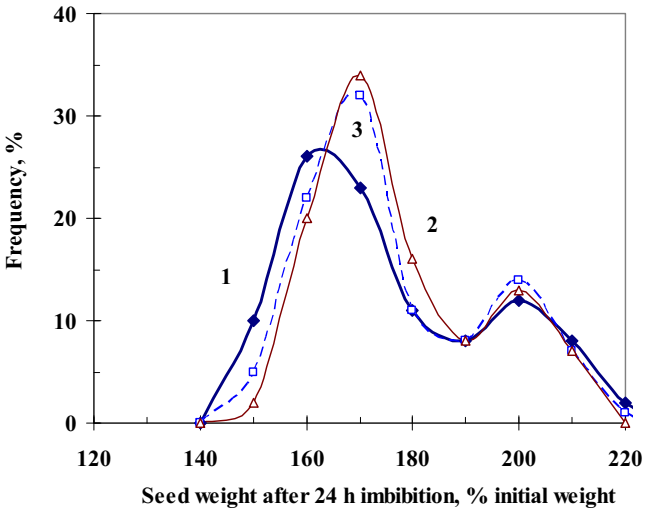

Fig. 4 Distribution of individual pea seeds (66% germinability) by weight gain after 24 h imbibition on filter paper moistened with water (1) and two antioxidants, carnosine (2) and propylgallate (3). For constructing each distribution 150 seeds were used.

Table 2 Influence of antioxidants on frequencies of normal and abnormal seedlings and nongeminated seeds, %.

Quality of seedling and nonprotruded seeds	Treatments		
	Tap water	Propylgallate	Carnosine
Normal seedlings	66 ± 5	83 ± 5	85 ± 6
Abnormal seedlings	25 ± 4	9 ± 3	6 ± 2
Suffocated seeds	6 ± 2	6 ± 2	6 ± 2
Dead seeds	3 ± 1	2 ± 1	3 ± 1

Note: A seed lot with 66 germination percentage was used. In all experiments, seeds were allowed to imbibe for 24 h in Petri dishes prior to their transfer to filter paper rolls for germination in the same solutions.

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