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Post-hypoxic Oxidative Stress in Aging Pea Seeds: I. Hypoxia Development during Imbibition

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ABSTRACT

During seed transition from quiescence to metabolic activity and subsequent germination, orthodox seeds become sensitive to stress. Aging seeds become particularly sensitive to stress conditions and during storage, seeds undergo aging that deteriorates their quality. Each seed lot contains strong seeds, weak seeds, and dead seeds, but dry seed quality cannot be assessed prior to germination. Nevertheless, air-dry pea seeds differing in germinability can be divided into three fractions using the method of room temperature phosphorescence (RTP): fraction I, alive high-quality seeds which will produce normal seedlings; fraction II, alive but weak seeds which will produce morphologically abnormal seedlings or will not germinate at all; and fraction III, dead seeds. The imbibition patterns of fraction I and fraction II seeds were compared during germination. The water uptake during imbibition was higher in the fraction II seeds than in those of fraction I. A higher respiration rate and greater limitation of oxygen diffusion by seed coats in fraction II seeds induced oxygen deficiency, which can be considered as hypoxic conditions for embryo. In order to assess the hypoxia level, we developed a non-intrusive luminescent method based on endogenous porphyrin phosphorescence of seeds. Result showed that hypoxia did not appear in fraction I seeds. In fraction II pea seeds, the hypoxia starts to develop after 12-16 h imbibition. When oxygen deficiency enhanced, alive seeds did not germinate as they died due to suffocation during imbibition. If the level of oxygen deficiency was not high (<50 arbitrary units (aU) of porphyrin phosphorescence) and radicles protruded, the emerging seedlings exhibit various morphological defects. Since hypoxia did not impair DNA replication (2C-4C) prior to radicle protrusion, it did not directly contribute to morphological abnormalities in seedlings.

Keywords: *Pisum sativum*, porphyrin phosphorescence, room temperature phosphorescence, seed aging, seed fractionation, seed quality
Abbreviations: aU, arbitrary units; **RH**, relative humidity; **RTP**, room temperature phosphorescence

INTRODUCTION

Orthodox seeds are seeds which survive natural dehydration or drying to low water content, when seeds have lost free water. It is commonly supposed that orthodox seeds do not experience stress, because air-dry seeds have extremely slow rate of metabolism. Moreover, owing to seed coats, seeds are protected from abrupt environmental changes. However, seed transition from quiescence to active metabolism and germination results in acquired sensitivity to stress conditions, the phenomenon being more evident in deteriorating seeds by age.

The quality of seeds declines during storage at various rates (Isely 1957), and any seed lot consists of a wide range of quality seeds, whish may be classified as strong and weak seeds (alive and able to produce normal or abnormal seedlings, respectively), and dead seeds, which do not germinate at all. Generally, air-dry as well as early imbibing pea seeds of different quality show no visible difference. Only at germination, the difference between normal and abnormal seedlings (seed quality) is visible (**Fig. 1**). According to ISTA (1996) if the radicle emerges the seeds are considered viable. If the radicle emergence does not occur after six days of imbibition the seeds may be considered as non-viable. The pea seeds are taken germinated if they produced normal seedlings after 6-day germination, whereas they are recorded non-germinated if they produced seedlings with morphological defects.

The method of room temperature phosphorescence (RTP) adopted here allowed us to separate any lot of low-quality air-dry seeds into three fractions comprising strong, weak and dead seeds (Veselova *et al*. 1999). Strong seeds of fraction I produced only normal seedlings. Weak seeds of fraction II either produced morphologically defective seedlings or did not germinate at all. Fraction III contained non-viable seeds.

The fractionation of air-dry pea seeds according to their room temperature phosphorescence has revealed the appearance of a weak seed fraction in the initially homogeneous seed lot after accelerated seed aging or after γ-irradiation. These seeds mostly produced abnormal seedlings with morphological defects (Veselova *et al*. 1999; Veselovsky *et al.* 2006).

Probably, morphological defects of seedlings were due to the impairment of cell divisions in the embryo axis (Roberts 1972; Priestley 1986; Vertucci 1989). During storage of air-dry orthodox seeds, the impaired cell division and appearance of abnormal seedlings may follow from: (a) lipid peroxidation (Smith and Berjak 1995); (b) irreversible non-enzymic reaction of lipid peroxidation product (malonic dialdehyde, MDA) or reducing sugars (glucose, galactose) with amino groups of membrane proteins and nucleic acids (amino-carbonyl reaction) (Sun and Leopold 1995; Murthy and Sun 2000; Murthy *et al*. 2003); and (c) higher permeability of cell membranes (Roberts 1972; Bewley and Black 1994; Golovina *et al*. 1997). Chromosome abberrations were not observed in dry seeds, but appeared in imbibing seeds (Roberts 1972).

During the transition from quiescence to imbibition cell division can be impaired by the following causes; (a) Imbibition stress. When the water flow into dry seeds is too rapid, the embryo membranes may loss their integrity, thereby causing the leakage of ions, amino acids, carbohydrates, and other substances. Upon the submergence of decoated pea seeds, some embryo cells die within few minutes or even seconds (Larson 1968; Duke and Kakefuda

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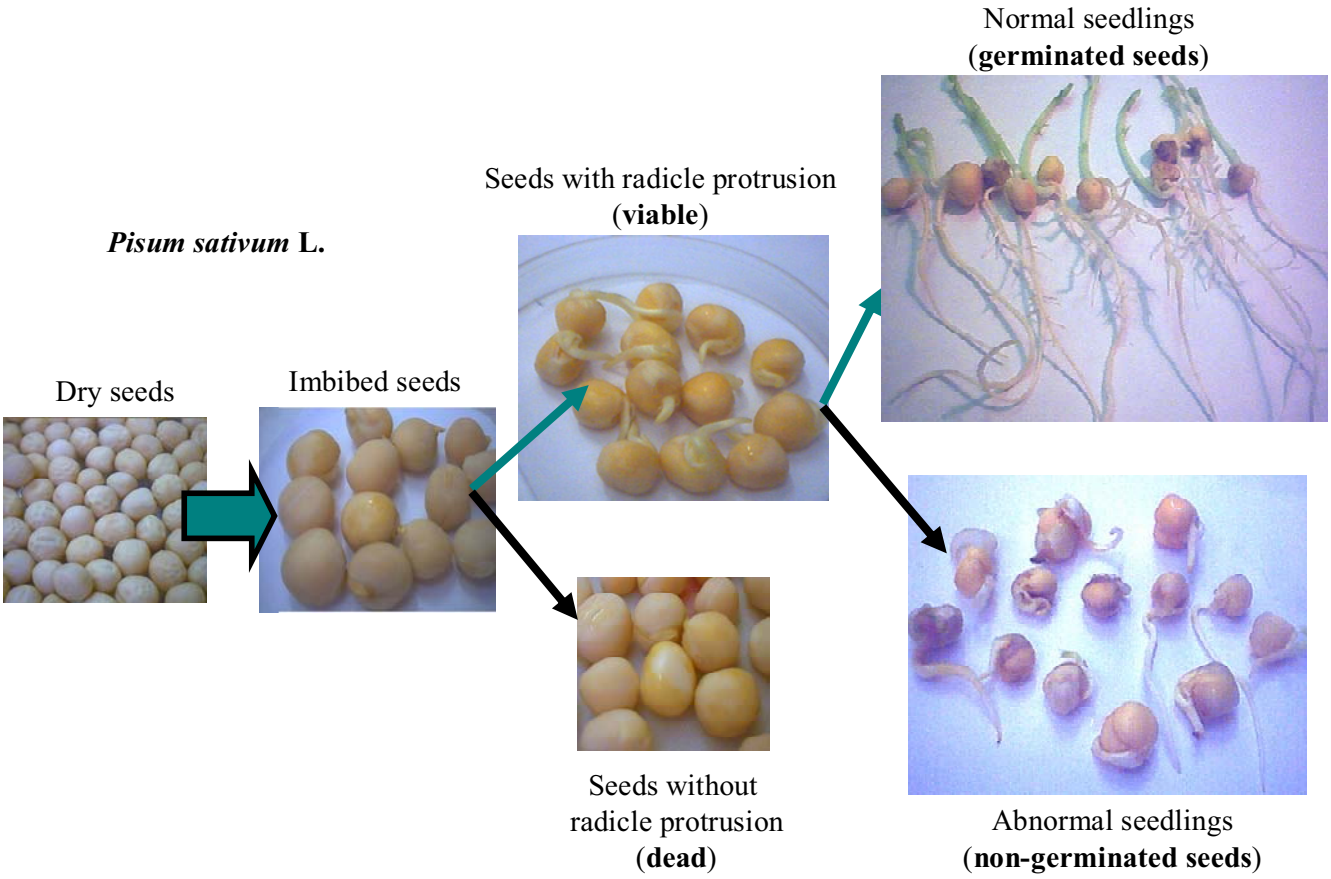

Fig. 1 A scheme of imbibing pea seed behavior at germination.

1981; Powell and Mattews 1978; Hoekstra *et al*. 2001). (b) Hypoxia stress. When the imbibition proceeds under excessive water content, the seeds, especially large seeds, suffer from oxygen deficiency. Toxic products of anaerobic fermentation inhibit seed germination (James 1953; Duke and Kakefuda 1981). (c) Post-hypoxic stress. Studies on hypoxic states in plant and animal tissues and cell cultures showed that the post-hypoxic aeration was even more dangerous than anoxia because of the so-called post-hypoxic oxidative stress (Crawford *et al*. 1994; Pfister-Sieber and Brandle 1994).

The aim of our work was to investigate the stress state of aging pea seeds during imbibition and germination. Experimental work was carried out to determine: (1) the effect of rapid water inflow into air-dry seeds, resulted in loss of viability; (2) the role of hypoxia in destroying the embryo axis cells during the imbibition; (3) possibility of development of eventual morphological defects in emerging seedlings due to hypoxia stress.

MATERIALS AND METHODS

Seed material

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

Germination and radicle protrusion testing

Seeds (four replicates of 25 seeds) were allowed to germinate at 20°C in filter paper rolls moistened with tap water. Radicle protrusion was recorded when the length of embryo axis organs in a germinating seed exceeded 4 mm. Seeds were defined as germinated if they produced normal seedlings after 6-day germination. Seedlings with morphological defects and various growth disturbances (seedlings with well-developed main root lacking epicotyl, with well-developed epicotyl lacking hypocotyl, with disturbed root growth due to impaired meristem or with two roots; with stubby, retarded and spindly roots) were classified as abnormal according to ISTA Rules (ISTA 1996). Following these rules, seeds must be considered non-viable if radicle emergence did not occur after six days of imbibition.

Seed imbibition

Seed imbibition rate was measured by the weighing method. Each seed blotted with filter paper was weighed after 0, 2, 4, 6, 8, 24 and 48 h imbibition. Seed fresh weight increment was expressed as the percentage of initial seed weight.

Seeds from fractions I, II and III (20 seeds in each) separated by RTP method (see results) were allowed to imbibe on a plastic tray (32 × 22 × 9 cm) at 20-22°C. A piece of glass (31 × 21 × 0.4 cm) was placed on the bottom of the tray on special plates (20 × 2 × 0.5 cm) so that it did not touch the bottom. The glass was covered with filter paper with a plastic grid (0.3 cm high, with cells of 2 × 2 cm) on it. Each seed was placed into individual cell. Seeds were covered with an additional layer of filter paper. Water was added into plastic tray in such a manner, that seeds did not touch the surface of free water. After 48 h imbibition, seeds were transferred to rolled towels which were placed in an upright position for germination test.

In order to study the imbibition damage, air-dried seeds were allowed to imbibe in Petri dishes (6 cm in diameter) between two layers of filter paper at 18°C. Four milliliters of water or 16.5% polyethylene glycol (PEG-6000, Serva, Germany) solutions (osmotic potential of -0.3 MPa) were added into each dish, containing 10 seeds. To reduce the imbibition damage in some experiments Petri dishes were placed in a thermostat at 26°C with seeds submerged with their lower side to a depth of 1-2 mm, or seeds were prehydrated at 18°C and 100% relative humidity (RH) for 48 h at room temperature and then allowed to imbibe in Petri dishes with water (4 ml) at 18°C. After 20 h imbibition, these seeds were transferred for germination into the filter paper rolls.

Oxygen uptake

Oxygen uptake by individual seeds (intact and without seed coat) or excised individual embryo axis was measured polarographically (L7 Polarograph, Laboratorni Pristroje, Praha) at 20°C, using a Clark-type electrode with a diameter of 20 μm and 8-μm thick film (E5047 PO_2 Electrode, Radiometer A/S, Denmark). An axis (7 mg) was incubated in the chamber with 60 μl water. In the case of intact or decoated seed, its side was took the place of the outward wall of this chamber (diameter 7 mm). The decline of oxygen partial pressure in the chamber during O_2 uptake was recorded in each seed (or axis) three times: after full depletion of oxygen in the chamber by respiring seed, a new portion of water was added to the chamber. Oxygen concentration was expressed as μMol/l. The rate of oxygen uptake was calculated from the slope of the oxygen pressure curve and expressed as μMol/(min·seeds). Experiments were conducted in five replicates.

RTP in air-dry seeds and phosphorescence of endogenous porphyrins in imbibing seeds

Phosphorescence was measured with a device as previously described (Veselova *at al*. 1999). The system consisted of double-disk phosphoroscope that provided intermittent illumination of a seed with 6 ms flashes (a KGM-100 incandescent lamp, Russia, illuminance of 60 klx). The seed phosphorescence was measured in the dark between recurrent flashes, within 3-18 ms after the light pulse. The phosphorescence of each seed was measured for 2-3 s with a FEU-79 photomultiplier tube (Russia) and expressed in arbitrary units.

Analysis of nuclear DNA amount

This analysis was carried according to Redfearn *et al.* (1995) with a modification. Two mm-long radicle tips were excised from the embryo axes. Nuclear samples were prepared by grinding five radicle tips in 0.7 ml of isolation buffer (0.2 M mannitol, 10 mM Mes-buffer, 10 mM NaCl, 10 mM spermine tetrahydrochloride, 2.5 mM Na_2-EDTA, 2.5 mM dithiothreitol, 0.05 v/v Triton X-100, pH 5.8). Then 10 μl of 5 μg/ml ethidium bromide solution was added to induce DNA fluorescence. The nuclear DNA content was measured 10 min after the ethidium bromide addition with a microfluorometric analyzer by placing 10 μl of suspension on a slide under a fluorescence microscope (LUMAM 13, Russia). The image was scanned with a video camera (QX3, Intel, USA) and analyzed with an original computer program GFAOC IAFC developed at Biophysics Department by V.B. Turovetsky and S.V. Galchuk. The average intensity of each stained nucleus and background were measured for 600-800 nuclei (25-80 nuclei per slide) at each experimental point. The fluorescence intensity was calculated as the difference between the intensity of each nucleus and background reading. The DNA content per nucleus was calculated from the intensity of nuclear fluorescence. The amount of 2C DNA nuclei was taken according to the nuclear fluorescence in dry pea seeds.

RESULTS AND DISCUSSION

Fractional composition of air-dried seed lots

Previous work (Veselovsky and Veselova 1990; Veselova *et al.* 1999) reported that air-dry seeds produce delayed light emission (room temperature phosphorescence, RTP) after exposure to white irradiation. Water reduces this emission. With water content increasing from 6 to 20% of seed fresh weight, the RTP intensity decreased linearly in semi-logarithmic coordinates. The RTP method permits detection of 0.1-0.2% difference in the equilibrium water content for each individual seeds in a seed sample. The close relationship between RTP and seed moisture content indicates that the distribution of seeds according to RTP reflects actually the distribution of seeds according to their water content.

In the lot of aged seeds with 48% germination, the distribution of air-dry seeds by RTP exhibits three maxima (**Fig. 2**), allowing to sort three fractions with different seed quality: viable seeds in the fractions I and II and dead seeds in fraction III, as previously reported by Veselova *et al.* (1999).

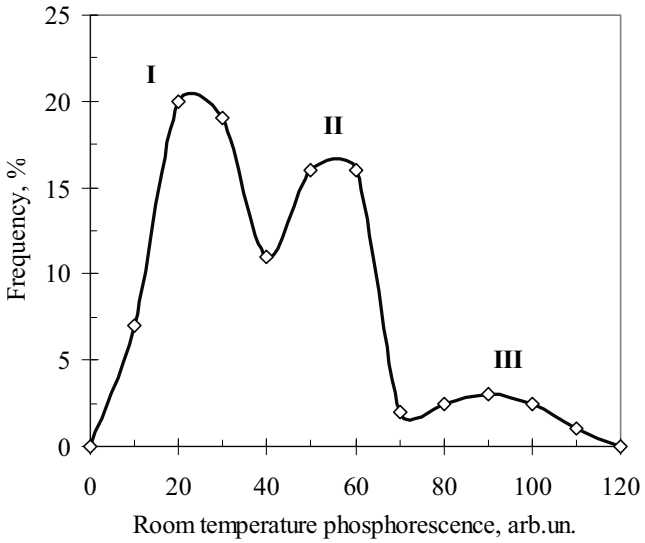

Fig. 2 Distribution of individual air-dry pea seeds (in the lot of 48% germinability) by the levels of room temperature phosphorescence (RTP). Each point is the mean of four replicates. I, II III indicate the fraction numbers.

When pea seeds germinated at 18°C between two layers of filter paper, the seeds of fraction I produced 96-98% normal seedlings. In the fraction II, 50-60% of seeds did not germinate at all; 35-40% of seeds produced seedlings with morphological defects, and the remaining 10-15% of seeds produced seedlings with normal *habitus*, although the length of their main roots was 2-3 times shorter than those from fraction I seeds. Fraction III seeds did not germinate.

In order to explain why the number of non-germinated seeds exceeds fraction III and why the viable seeds of fraction II did not germinate water uptake rate, respiration rate, and RTP changes were determined during the imbibition of each sorted fraction seeds. Samples of seeds from fraction I (RTP intensity 25 ± 5 aU, moisture content 9.8% fresh weight), fraction II (50-60 aU, moisture content 8.9%), and fraction III (80-100 aU, moisture content 8.2%) were analyzed. Attemps were also made to clarify at which stage – during storage of dry seeds or at a certain step of germination – the normal morphogenesis becomes to be impaired resulting in morphologically defective seedlings.

Phosphorescence of imbibed seeds

When intact air-dry pea seeds were placed on wet filter paper, their RTP dropped to a background level within 2-h imbibition (**Fig. 3**, the emission decrease to the left from dotted line). However, when seeds were kept on filter paper for a longer period (10-12 h), the emission of imbibing seeds appeared (**Fig. 3**, the emission to the right from dotted line). This emission recovery was characteristic of fraction II seeds and was not observed in fraction I seeds. Later on, the emission increased with time; the higher was ambient temperature, the faster was the increasing (Veselova *et al*. 2003). Dead seeds of fraction III did not contribute to light emission.

The strong seeds from fraction I showed either no emission or it was very weak, which disappeared after the embryo radicle penetrated through the seed coat (**Fig. 3**, curve 1).

When the fraction II seeds imbibed for 24-48 h, the emission of some seeds increased five-fold or even higher as compared to RTP of air-dry seeds. In this case the seed softened and then lysed (curve 3). If the embryonic radicle of fraction II seeds was able to protrude through the seed coat within 40-42 h of imbibition, the emission declined

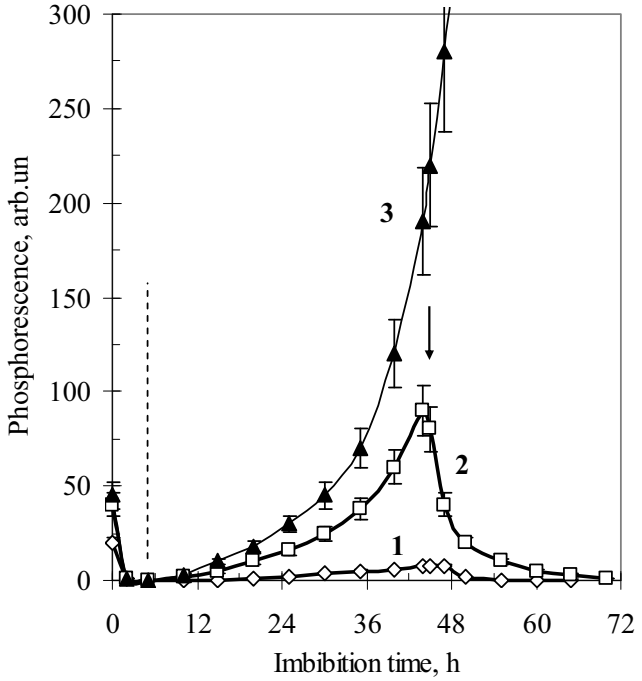

Fig. 3 Time course of porphyrin phosphorescence in fractions I and II of pea seeds during imbibition. Four replicates of 10 seeds were used for each test. 1, 2 and 3 – see text. Arrow indicates radicle protrusion.

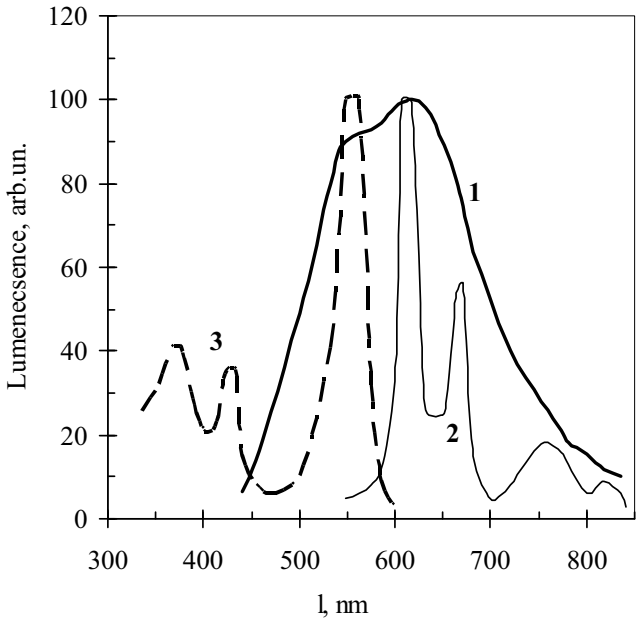

Fig. 4 Phosphorescence spectra. 1) Room temperature phosphorescence of air-dry pea seeds; 2) Emission spectrum of imbibing pea seeds; 3) Excitation spectrum of imbibing pea seeds.

slowly (curve 2). However, such seeds usually produced morphologically abnormal seedlings. These data suggested that the increase in emission of fraction II imbibing seeds indicates the deterioration and loss of viability.

Imbibing seed emission spectra

In order to elucidate the nature of the imbibing seed emission, we compared its spectral characteristics with those of RTP of air-dry seeds. The RTP spectra had a broad band in the 400-800 nm range without clear structure (**Fig. 4**, curve 1) (Veselova *et al*. 1985). Hence, this emission seems to be caused by several sources. The emission spectrum of imbibing seeds occupies the red region and has four pronounced maxima (curve 2). The excitation spectrum has three maxima at 360, 420 and 560 nm (curve 3). The emission and excitation spectra of imbibing seeds exhibited a typical band structure specific for phosphorescence of metal-free porphyrins (pheophorbid). Apparently, these endogenous seed porphyrins are the products of chlorophyll degradation (Matile *et al*. 1999; Eckardt 2009).

Porphyrin phosphorescence as a measure of hypoxia

The phosphorescence of biopolymers is usually observed at cryogenic temperatures. At temperatures above 170 K, phosphorescence declines quickly because of oxygen diffusion that quenches molecular excitations (Guillet 1987). The RTP of air-dry seeds can be detected because the oxygen content inside seeds is very low. After seed moistening, the oxygen diffusion into the seeds increases and phosphorescence declines.

Porphyrin phosphorescence can be detected only at low oxygen concentration (Terenin 1967). Thus, the emission originates only from those seed parts that undergo oxygen deficiency. Previous work (Veselova *et al*. 1988) reported that moistened soybean seed coats, cotyledons, and embryo axes did not emit light in the atmospheric air. The emission of intact soybean seeds disappeared upon the wounding of seed coat. After the emission disappeared by seed coat impairment, it could be recovered by subsequent transfer of seeds into a nitrogen atmosphere or oxygen-free water to which Na_2SO_3 was added. In deoxygenated media all seed parts emitted phosphorescence. Thus, the phosphorescence of intact imbibing soybean seeds is due to oxygen deficiency arising under the seed coat.

In imbibing pea seeds, the hypoxia apparently developed not only under the seed coat, but also in the embryo cells. When an imbibing pea seed showing porphyrin phosphorescence intensity of 245 aU was decoated, the level of porphyrin phosphorescence decreased to 150 aU (**Table 1**). When the cotyledon was fragmented, the phosphorescence declined to 100 aU, but the complete phosphorescence disappearance was observed only after seed homogenization. The homogenate emission reappeared under anoxic condition (in the presence of Na_2SO_3).

The oxygen deficiency in imbibing legume seeds is one of the injuring factors during germination (Duke and Kakefuda 1981). Hypoxia usually arises because the rapid oxygen consumption by seed embryo is not counterbalanced by oxygen diffusion through the seed coat that acts as a hardly-permeated barrier for water and oxygen transport toward the embryo (James 1953; Larson 1968; Leblova 1978; Al-Ani *et al.* 1985).

The decrease in oxygen concentration inside a sealed chamber due to the cytochrome-mediated respiration of embryo axes led to the proportional increase in porphyrin phosphorescence (**Fig. 5**). Inhibition of mitochodrial respiration by cyanide deccelerated oxygen consumption and was accompanied by an increase in porphyrin phosphorescence (Veselova *et al*. 1988). As a result, the phosphorescence intensity of endogenous porphyrins was used as a marker of oxygen deficiency in imbibing seeds.

Table 1 Porphyrin phosphorescence in intact pea seed and its parts.

	Porphyrin phosphorescence (aU)
Intact imbibing seed	245 ± 18
Seed coat	0
Embryo (decoated seed)	150 ± 12
Cotyledon	100 ± 8
Crushed cotyledon	60 ± 5
Homogenized cotyledon	0

aU = arbitrary units

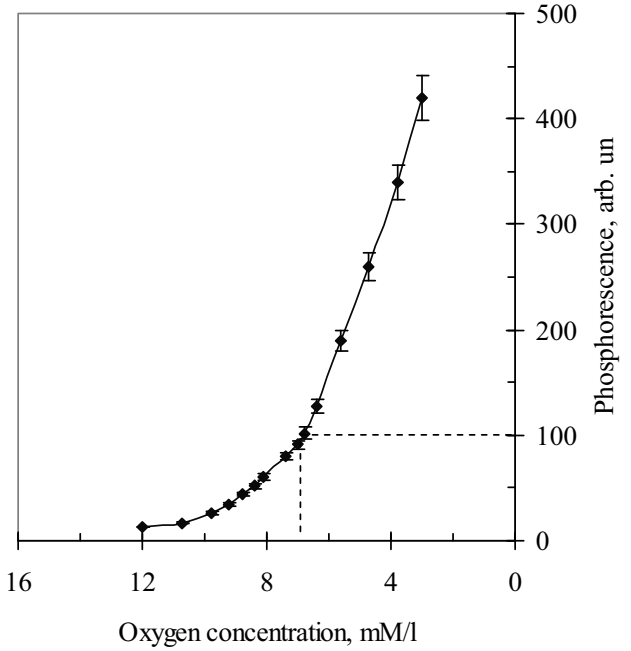

Fig. 5 Correlation between changes in oxygen concentration and level of porphyrin phosphorescence during respiration of embryo axis in sealed chamber.

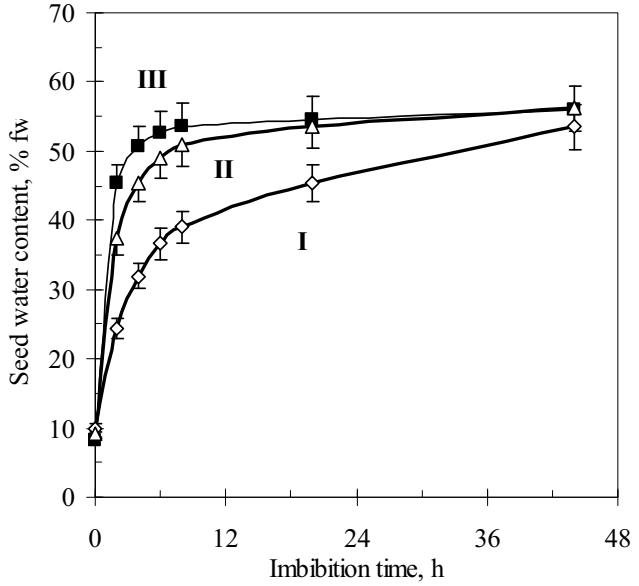

Fig. 6 Imbibition kinetics of seeds belonging to different fractions. The fraction designations (I-III) are indicated by figures near the curves. Each point is the average fresh weight of 20 seeds.

Why does hypoxia arise in weak seeds of fraction II but does not appear in fraction I seeds?

To answer this question, the rates of water uptake and oxygen consumption were estimated in seeds from each sorted fractions.

The rate of water uptake during seed imbibition was two-fold higher for fraction II seeds compared to fraction I (**Fig. 6**). In dead seeds of fraction III, the water uptake rate was even higher. Since the seed coat is the main barrier on the water absorption likely it retarded the water flow into seeds of fractions I and II to aproximately equal extent. Therefore, various rates of water uptake by seeds of fractions I and II might reflect different states of water channels formed by aquaporins rather than variations in permeability of seed coats (Veselova and Veselovsky 2006).

The appearence of free water in imbibing seeds was accompanied by activation of oxygen consumption. The rate of oxygen consumption reached a steady-state level when the pea seed moisture content approached 30-35%. After completion of mitochondrial biogenesis at pea seed moisture content of 45% (Obroucheva 1999), the rate of oxygen uptake increased again, and the affinity of respiration to oxygen became higher. **Fig. 7** shows oxygen uptake rates as a function of pO_2 for intact (I and II) and decoated (I' and II') seeds from fractions I and II after 20-h imbibition. At this stage of imbibition, the water content was 43% for fraction I seeds; 50%, for fraction II seeds. This means that a higher rate of oxygen uptake by fraction II seeds, as compared to fraction I seeds, was due to the completion of mitochondriogenesis and fully-developed cytochrome respiration.

Our measurements of oxygen uptake by intact and decoated seeds showed that the seed coat in fraction I seeds reduced oxygen uptake by factor of 2.1 (15 and 32 μmol O_2 $min^{-1}seed^{-1}$ with and without seed coat, respectively) (**Fig. 7**). The seed coat of fraction II seeds reduced oxygen uptake by factor 2.6 (29 and 76 μmol O_2 $min^{-1}seed^{-1}$ with and without seed coat, respectively). Apparently, the oxygen deficiency in the fraction II seeds was due to a higher oxygen uptake rate by the seed embryo and to slower oxygen diffusion through the seed coat in fraction II seeds.

The rate of oxygen uptake by dead seeds of fraction III was close to zero (**Fig. 7**, curve III). Since porphyrin phosphorescence did not increase in these seeds consequently,

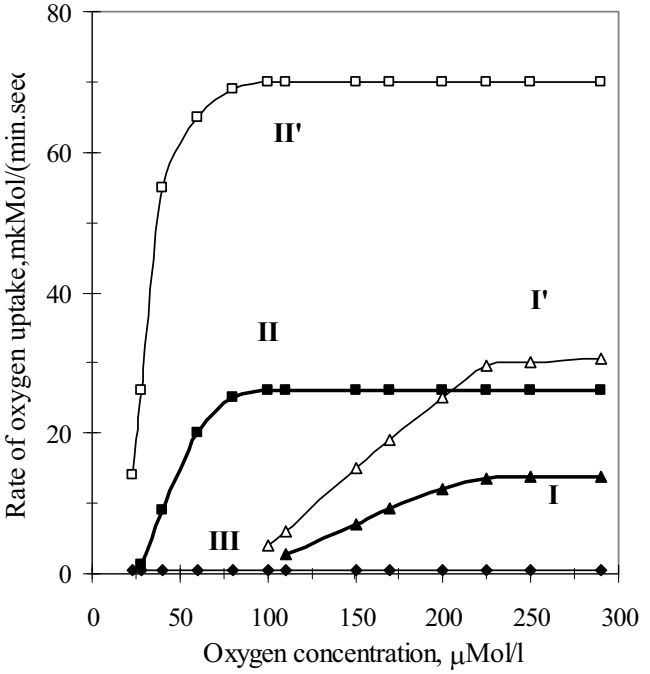

Fig. 7 Effect of oxygen concentration on the rate of oxygen uptake by intact (I and II) and decoated (I' and II') seeds from fractions I and II and seeds from fraction III. Five seeds were measured for each curve.

there was no oxygen deficiency under the seed coat. The absence of porphyrin phosphorescence in seeds without radicle protrusion provides the mean to differentiate dead seeds from suffocated seeds having high level of porphyrin phosphorescence.

Hypoxia and the primary imbibition injuries

Table 2 shows the influence of imbibition conditions on frequencies of normal and abnormal seedlings and on frequency of non-germinated (suffocated and dead) pea seeds. When the seeds germinated at 18°C between two layers of filter paper without direct contact with water, 48% of seeds produced normal seedlings (**Table 2**). This percentage of germinated seeds corresponded to the number of air-dry seeds in fraction I, as follows from seed distribution by RTP (**Fig. 2**). The amount of dead seeds coincided with

Table 2 Effect of various imbibition conditions on the correlations (%) between normal and abnormal seedlings and seeds without emergence.

Quality of seedlings and seeds without radicle emergence	Imbibition between two layers of filter paper* at 18°C	Imbibition in direct contact with water at 18°C and 26°C		Preexposure of seeds at 18°C and 100% RH to increase moisture content to 25%	Imbibition for 20 h in PEG solution at 18°C
Normal seedlings	48 ± 4	40 ± 3	47 ± 4	47 ± 4	68 ± 5
Abnormal seedlings	19 ± 4	15 ± 4	19 ± 4	20 ± 3	14 ± 4
Suffocated seeds	25 ± 4	27 ± 5	25 ± 4	24 ± 4	10 ± 4
Dead seeds	8 ± 3	18 ± 3	9 ± 3	9 ± 3	8 ± 2

Notes: A seed lot with a 48% germination percentage was used. (*) Imbibition in the absence of direct contact with bulk water. In all other treatments, seeds were allowed to imbibe for 20 h in Petri dishes prior to their transfer to filter paper rolls for germination.

Table 3 DNA content (% of total DNA) and 4C/2C ratio in cell nuclei from imbibing pea seed belonging to three fractioms.

Imbibition time, h	Fraction I			Fraction II			Fraction III		
	2C	4C	4C/2C	2C	4C	4C/2C	2C	4C	4C/2C
0	88.0	12.0	0.14	88.0	12.0	0.14	91	9.0	0.1
6	84.7	15.3	0.18	82.6	17.4	0.21	90	9.0	0.1
24	75.4	23.6	0.30	71.4	28.8	0.40	90	10.0	0.11
48	56.4	39.5	0.70	54.0	46.0	0.85	90	10.0	0.11
72	30.7	63.0	**2.02**	53.5	46.5	**0.87**	89	11.0	0.12

the number of fraction III seeds. The total number of suffocated seeds and morphologically defective seedlings corresponded to the number of fraction II seeds.

When pea seeds germinated at 18°C in Petri dishes with their lower side submerged in water by 1-2 mm, the number of normal and abnormal seedlings decreased (to 40 and 15%, respectively) (**Table 2**). Based on the level of porphyrin phosphorescence we concluded that, among 45% of seeds showing no radicle protrusion, 27% of seeds died of suffocation and the remaining 18% were dead seeds. Clearly, in fraction I, not all the seeds produced normal seedlings under given germination conditions. Considering the increase in the number of dead seeds, it is reasonable to assume that the additional lethality of seeds were due to stress injury at the first stage of seed hydration.

The imbibition injury of seeds can be diminished by several means, such as (a) germination at elevated temperature (26°C), (b) preexposure of seeds at 18°C and 100% RH for 2-3 days, or (c) the suppression of water uptake by submerging seeds in osmotic media (Powell and Matthews 1978; Duke and Kakefuda 1981; Woodstock and Tao 1981; Vertucci 1989; Zeng *et al.* 1998).

When pea seeds germinated at 26°C in Petri dishes with their lower side submerged in water by 1-2 mm or germinated after preincubation at 18°C and 100% relative humidity (RH), which elevates the seed moisture content to 25-27%, the control germination percentage was restored. The number of dead seeds decreased reaching the level observed upon germination between two layers of filter paper (in the absence of immediate contact with water). Thus, seed germination under these conditions was beneficial, as it avoided the primary damage occurring upon the immediate contact of air-dry seed with bulk water.

When pea seeds were allowed to imbibe in PEG-6000 solution for 20 h, the rate of water uptake decreased nearly two-fold. Following this treatment, the percentage of dead seeds was as low as upon germination between two layers of filter paper. Moreover, the number of suffocated seeds and abnormal seedlings decreased in this case, whereas the number of normal seedlings increased. Hence, the retarded water uptake by seeds submerged into osmotic media not only eliminated the lethal injuries at the first stage of seed hydration, but also prevented the development of oxygen deficiency in fraction II seeds. As a result, some seeds from the fraction II produced normal seedlings, and the germination percentage of a seed lot increased.

DNA replication

It is known that the DNA reparation phase precedes the DNA replication (Osborne 1983; Smith and Berjak 1995). Morphologically defective seedlings development is due to impairment of cell division related to DNA damage. The cell division in embryo axes of pea seeds commences few hours after the radicle protrusion. For this reason, DNA replication was examined at the stage of preparation of embryo axis cells for division. The vast majority of cell nuclei in air-dry seeds contain 2C DNA, because the cell cycles are arrested at G_1 or G_0 stage during seed maturation (Obroucheva 1982; Bino *et al*. 1993; Redfearn *et al*. 1995; Gornik *et al.* 1997). In air-dry pea seeds of fractions I, II and III, approximately 90% of nuclei contain 2C DNA (**Table 3**). During seed imbibition, the 2C DNA content decreased, while the frequency of 4C DNA-containing nuclei increased. The dynamics of this process is evident from the changes in 4C/2C ratio.

There were no changes in DNA profile of fraction III dead seeds. The content of 2C DNA did not decrease, and DNA replication did not occur. The 4C/2C ratio increased in both fractions I and II during the first 48 h of imbibition. Moreover, this ratio rose faster in the fraction II seeds which exhibited higher rates of water uptake.

However, after the radicle protrusion at 44-48 h of imbibition, the 4C/2C ratio did not grow in fraction II seeds, whereas this ratio continued to increase in fraction I seeds. Without any doubt, the difference in 4C/2C ratio between the seed fractions I and II is confident (at this imbibition time). The retardation of DNA replication occurred just before or immediately after the radicle protrusion. This retardation is unlikely related to the onset of mitotic cycle, because no mitoses were observed at this stage in fraction II seeds; only cell elongation took place. The retardation of DNA replication might be caused by fermentation products arising in fraction II seeds under hypoxic conditions during the imbibition. However, the oxygen deficiency in pea seeds of fraction II arose after 12- to 20-h of imbibition and persisted until the radicle protrusion at 40-46 h of imbibition. Nevertheless, the occurrence of DNA replication at this stage provides evidence that the arrest of DNA replication in fraction II seeds could not be caused by hypoxia.

CONCLUSION

The appearance during germination of morphologically abnormal seedlings is one of earliest signs of seed aging, which indicates the impairment of cell division in the meristems of embryo axis (Isely 1957; Roberts 1972; Priestley 1986; Vertucci 1989). Using the RTP method we showed that morphologically abnormal seedlings arise from a discrete fraction of the air-dry seeds, termed fraction II (Veselova *et al*. 1999). In order to clarify whether injuries impairing cell division arise during seed storage or seed germination, we compared the imbibition and germination patterns, as well as DNA replication between seeds of fractions I and II.

The DNA replication during seed germination occurs

after repair process of the defects accumulated during storage of dry seeds (Osborne 1983). The DNA replication in fraction II seeds started synchronously to that in fraction I seeds. This indicates that air-dry seeds of fraction II did not contain unrepaired DNA lesions which might account for morphological defects in seedlings.

It is known that almost all seeds (especially large seeds of legumes) experience oxygen deficiency during germination under flooding (James 1953; Duke and Kakefuda 1981; Visser *et al.* 2003; Vartapetian 2005; Sachs and Vartapetian 2007; Vartapetian *et al.* 2008; Magneschi and Perata 2009). Our measurements of endogenous porphyrin phosphorescence in intact pea and soybean seeds revealed that some seeds experience oxygen deficiency even in the absence of direct contact with bulk water. Moreover, the hypoxia development occurred most frequently in the fraction II seeds (Veselova *et al*. 1988, 2003).

A new non-invasive method allowed us to assess hypoxia extent by testing the level of porphyrin phosphorescence. We found that the increase in porphyrin phosphorescence above 100 aU, reflecting the drop in oxygen concentration in pea embryo to 6-8 μM (**Fig. 4**), was a clear indication that the seeds suffered from hypoxic stress. These seeds suffocated during the imbibition, did not show radicle protrusion, and perished on the 5th to 6th days of imbibition.

At milder hypoxia level (porphyrin phosphorescence <80-100 aU), the embryo radicle was able to protrude through the seed coat, but related seedlings exhibited varios morphological defects.

In spite of hypoxia development in fraction II seeds with porphyrin phosphorescence level of 20-80 aU, DNA replication was shown to occur normally. Hence, hypoxia could not be responsible for DNA replication injury. The DNA replication in fraction II seeds ceased after the radicle protrusion, when the oxygen availability for embryo was restored.

The number of dead seeds and seeds without radicle protrusion was found to increase during stress imposed by either the primary contact of air-dry seeds to water or by severe hypoxia. Therefore, we propose that the post-hypoxic oxidative stress in pea seeds of fraction II was the only cause for DNA post-germinative cell division impairment in embryo axes, resulting in the appearance of morphologically abnormal seedlings.

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REFERENCES

Al-Ani A, Bruzau F, Raymond Ph, Saint-Ges V, Leblanc JM, Pradet A (1985) Germination, respiration and adenylate energy charge of seeds at various oxygen partial pressures. *Plant Physiology* **79**, 885-890

Bewley JD, Black M (1994) *Seeds, Physiology of Development and Germination*, Plenum Press, NY, 445 pp

Bino RJ, Lantery S, Verhoeven HA, Kraak HL (1993) Flow cytometric determination of nuclear replication stage in seed tissues. *Annals of Botany* **72**, 181-187

Crawford RMM, Walton JC, Wollenweber-Ratzer B (1994) Similarities between post-ischaemic injury to animal tissues and post-anoxic injury in plants. *Proceedings of the Royal Society of Edinburg* **102B,** 325-332

Duke SH, Kakefuda G (1981) Role of the testa in preventing cellular rupture during imbibition of legume seeds. *Plant Physiology* **67**, 449-456

Eckardt NA (2009) A new chlorophyll degradation pathway. *The Plant Cell* **21**, 700

Golovina EA, Tikhonov AN, Hoekstra FA (1997) An electron paramagnetic resonance spin-probe study on membrane-permeability changes with seed ageing. *Plant Physiology* **114**, 383-389

Gornik K, de Castro RD, Liu Y, Bino RJ, Groot SPC (1997) Inhibition of cell division during cabbage (*Brassica oleracea* L.) seed germination. *Seed Science Research* **7**, 333-340

Guillet J (1987) *Polymer Photophysics and Photochemistry. An Introduction to The Study of Photoprocesses in Macromolecules*, Cambridge University Press, UK, 391 pp

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. *Trends in Plant Science* **6**, 431-438

Isely D (1957) Vigor tests. *Proceedings of the Association of Official Seed Analysts* **47**, 176-182

ISTA (International Seed Testing Association) (1996) International Rules for Seed Testing: Rules 1996. *Seed Science and Technology* **24 (Suppl.)**, 29-156

James WO (1953) *Plant Respiration*, Clarendon Press, Oxford, 282 pp

Larson A (1968) The effect soaking pea seeds with or without seed coats has on seedling growth. *Plant Physiology* **43**, 255-259

Leblova S (1978) Pyruvate conversions in higher plants during natural anaerobiosis. In: Hook DD, RMM Crawford (Eds) *Plant Life in Anaerobic Environments*, Ann Arbor Science, Michigan, pp 155-168

Magneschi L, Perata P (2009) Rice germination and seedling growth in the absence of oxygen. *Annals of Botany* **103**, 181-196

Matile Ph, Hörtensteiner S, Thomas H (1999) Chlorophyll degradation. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 67-95

Murthy UMN, Kumar PP, Sun WQ (2003) Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. *Journal of Experimental Botany* **54**, 1057-1067

Murthy UM, Sun WQ (2000) Protein modification by Amadori and Maillard reactions during seed storage: role of sugar hydrolysis and lipid peroxidation. *Journal of Experimental Botany* **51**, 1221-1228

Obroucheva NV (1982) Seed germination. In: AA Prokofiev (Ed) *Seed Physiology*, Nauka (Science), Moscow, pp 223-274 (in Russian)

Obroucheva NV (1999) *Seed Germination: A Guide to the Early Stages*, Backhuys Publishers, Leiden, 158 pp

Osborne DJ (1983) Biochemical control systems operating in the early hours of germination. *Canadian Journal of Botany* **61**, 3568-3577

Pfister-Sieber M, Brandle R (1994) Aspects of plant behaviour under anoxia and post-anoxia. *Proceedings of the Royal Society of Edinburgh* **102B**, 313-324

Powell AA, Matthews S (1978) The damaging effect of water on dry pea embryos during imbibition. *Journal of Experimental Botany* **20**, 1215-1229

Priestley DA (1986) *Seed aging: Implication for seed Storage and Persistence in Soil*, Comstock Publications Association, Ithaca, 304 pp

Redfearn M, Clarke NA, Osborn DJ, Halmer P, Thomas TH (1995) DNA integrity and synthesis in relation to seed vigour in sugar beet. In: Ellis RH, Black M, Murdoch AJ, Hong TD (Eds) *Basic and Applied Aspects of Seed Biology*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 413-420

Roberts EH (1972) Cytological, genetically and metabolic changes associated with loss of viability. In: Roberts EH (Ed) *Viability of Seeds*, Chapman and Hall, London, pp 253-306

Sachs MM, Vartapetian BB (2007) Plant anaerobic stress I. Metabolic adaptation to deficiency. *Plant Stress* **1**, 123-135

Smith MT, Berjak P (1995) Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. In: Kigel J, Galibi G (Eds) *Seed Development and Germination*, Marcel Dekker Inc., NY, pp 701-746

Sun WQ, Leopold AC (1995) The Maillard reaction and oxidative stress during ageing of soybean seeds. *Physiologia Plantarum* **94**, 94-104

Terenin TN (1967) *Fotonika molekul kracitelei* (Fotonic of dyers moleculars), Nauka (Science), Leningrad, 308 pp (in Russian)

Vartapetian BB (2005) Plant anaerobic stress as a novel trend in ecological physiology. biochemistry, and molecular biology: 1. Establishment of a new scientific discipline. *Russian Journal of Plant Physiology* **52**, 826-844 (English translated)

Vartapetian BB, Sachs MM, Fagerstedt KV (2008) Plant anaerobic stress II. Strategy of avoidance of anaerobiosis and other aspects of plant life under hypoxia and anoxia. *Plant Stress* **2**, 1-19

Vertucci CW (1989) The Kinetics of seed imbibition: controlling factors and relevance to seedling vigour. In: *Seed Moisture*, Crop Science Society of America, Special Publication. **14**, 93-115

Veselova TV, Veselovsky VA (2006) Possible involvement of aquaporins in water uptake by pea seeds differing in quality. *Russian Journal of Plant Physiology* **53**, 96-101 (English translated)

Veselova TV, Veselovsky VA, Krasnovsky AA Jr., Lichsteld K (1985) Delayed luminescence of seeds. *Russian Journal of Biophysics* **30**, 711-712 (in Russian)

Veselova TV, Veselovsky VA, Kozar VI, Rubin AB (1988) Delayed luminescence of soybean seeds during swelling and accelerated ageing. *Seed Science and Technology* **16**, 105-113

Veselova TV, Veselovsky VA, Leonova EA (1999) What are the implications of changes in the heterogeneity of a seed population upon accelerated ageing? *Russian Journal of Plant Physiology* **46**, 477-183 (English translated)

Veselova TV, Veselovsky VA, Usmanov PD, Usmanova OV, Kozar VI (2003) Hypoxia and imbibition injuries to aging seeds. *Russian Journal of Plant Physiology* **50**, 835-842 (English translated)

Veselovsky VA, Veselova TV (1990) *Luminescencia rastenii. Teoreticheskie I prakticheskie aspekti (Plant Luminescence. Theoretical and Practical As-*

pects), Nauka (Science), Moscow, 200 pp (in Russian)

Veselovsky VA, Veselova TV, Korogodina VL, Florko BV, Mokrov YuV (2006) Bimodal changes in germinability of pea seeds under the influence of low doses of γ-radiation. *Radiation Biology. Radioekology* **46**, 691-696 (in Russian)

Visser EJW, Voesenek LACJ, Vartapetian BB, Jackson MB (2003) Flooding and plant growth. *Annals of Botany* **91**, 107-109

Woodstock LW, Tao KLJ (1981) Preventing of imbibition injury in low vigour soybean embryonic axes by osmotic control of water uptake. *Physiology Plantarum* **51**, 133-139

Zeng X-Y, Chen R-Z, Fu J-R, Zhang X-W (1998) The effect of water content during storage on physiological activity of cucumber seeds. *Seed Science Research* **8**, 65-68