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# INTEGRITY OF NUCLEAR DNA AND PHYSIO-BIOCHEMICAL INDICATORS OF *Pisum sativum* L. SEEDS UNDER ACCELERATED AGING

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Abstract

Currently, much attention is paid to understanding the roles of DNA and the main mechanisms for ensuring stability of the genome in maintaining seed viability during aging. It is also shown that significant oxidative damage to DNA occurs during seed swelling, and active DNA restoration processes are a factor that facilitates the initiation of DNA replication and rapid germination of seeds. Our objective was to study, on the example of two pea varieties and their hybrid, the effect of accelerated seed aging on the level of DNA damage (by DNA comet method) and biochemical indicators (lipid peroxidation, peroxidase activity, content of low-molecular antioxidants) in cells of embryos during seed swelling to find a relationship between these parameters and the changes in physiological parameters of seed germination. It is shown that accelerated aging leads to changes in pea seed germination capacity which are varietal specific, as well as in the biochemical indicators studied. The least resistant to the accelerated aging was Melkosemyannyi 2 variety, and the seeds of Saryal variety were medium-resistant. The seeds of a hybrid of these varieties were the most resistant which may be due to the effect of heterosis. Seed aging causes a significant increase in DNA damage assessed as DNA per cent in the tail of the comet and/or atypical comets. The longer the seeds were under aging conditions, the higher was DNA fragmentation in cells of the embryos upon swelling. Under 24 weeks of accelerated aging, there was a 1.6-3.3 % increase in DNA found in the tail of the comet, and the number of atypical comets in the embryo cells increased 17-40-fold depending on the variety (hybrid) as compared to control. Probably, a significant reduction of seed physiological parameters was caused by higher degree of nuclear DNA fragmentation, decreased enzymatic antioxidants activity (in particular, activity of peroxidases) and intensified oxidation in embryos. Intensification of oxidative processes is expressed as a 2.5-fold excess of lipid peroxidation in germs of a rapidly aging variety which is accompanied by low seed germination. It is assumed that the increase in the degree of DNA damage is a consequence of the depletion of antioxidant and repair enzymes and indicates a slowdown or lack of regenerative processes in the embryos of aging seeds.

Keywords: *Pisum sativum*, pea, seeds, accelerated aging, germination energy, lipid peroxidation, peroxidase, low-molecular antioxidants, DNA comet

The seeds' property to stay in a state of physiological dormancy for a long time, to endure adverse conditions while maintaining the viability, and to germinate successfully is the necessary condition for the beginning of the plant's life cycle and subsequent reproduction [1, 2]. It is considered that the preservation of seeds' viability for a certain time largely depends on the genetic characteristics of the species, as well as on the storage conditions. Among the factors influencing on the rate of seeds' aging, the temperature and humidity are con-

sidered the most significant [3, 4]. In addition, the longevity of seeds is conditioned by their quality which, in turn, is determined by the growing and ripening conditions, size of seeds, etc.

However, a long-term storage inevitably leads to the delay in sprouting, reducing of germination or to the complete loss of viability [5, 6]. The main causes of seeds' aging and death include the excessive formation of reactive oxygen species (ROS), inactivation of enzymes, destruction of proteins and lipids, breach of the membranes' integrity and degradation of DNA [6-9].

Currently, much attention is paid to investigating of the role of damaging DNA and of the main mechanisms of maintaining the genome's stability in the preserving of the seeds' viability while aging. The experimental material confirming the intensification of the processes of oxidative damage to DNA while the seeds swelling even their quality is high is being accumulated, and the actively going processes of DNA recovery are considered as a factor contributing to the initiation of replication and to the rapid germination of seeds [10]. The most common damages to the DNA molecule are single-strand and double-strand breaks, as well as the changes in the structure of purine and pyrimidine bases. The slowdown of sprouting and reduction of germination of aging seeds are deemed to be related to the long-term repair of DNA and delayed replicative synthesis of DNA [11, 12].

The investigation of the seeds' physiological and biochemical characteristics, predicting their longevity and storability are mainly performed using the methods of accelerated aging under the conditions conducive to increasing the seeds' moisture and while exposing to high temperature [13, 14]. Under the influence of precisely these factors, as a rule, seeds lose their viability in a short time.

The investigation of the interrelation between the embryo genome's integrity and seeds' quality is of great interest. However, the information on the quantitative assessment of the degree of damage to DNA and its influence on the seeds' germination capacity available in the scientific literature is quite poor. This is probably due to the little experience in application of the DNA comet method on plants while it is widely and efficiently used in the in vivo and in vitro systems for evaluating the genotoxic effects when affecting of various factors of physical and chemical nature on human and animal cells and on microorganisms.

In our information release, we described the genotoxic effects and functions of prooxidant and antioxidant systems accompanying the seeds' aging in two varieties of edible pea (*Pisum sativum* L.) and their intervarietal hybrid, which is important for understanding the physiologic-biochemical changes and for predicting the recovery processes under the conditions of long-term storage. As the result, we have not only confirmed the fact of damages to DNA, but also described them quantitatively, which partially completes the lack of information about the role of damage to DNA in the seeds' aging.

The objective of this work is to investigate the influence of accelerated aging of pea seeds on the indices of their viability, oxidative processes and stability of the nuclear DNA structure in the embryos' cells while germination (in the swelling phase).

*Techniques.* The seeds of three samples (Saryal and Melkosemyannyi 2 varieties and their hybrid Saryal × Melkosemyannyi 2) of edible pea (*Pisum sativum* L.) have been gotten in the conditions of the experimental station located on the valley side of the Lena river's middle reach. The plot's soil is taiga cryogenic, yellowish, solodized soil, typical for the agricultural zone of Central Yakutia. The growing season has been assessed as arid (HTI [hydrothermal index] = 0.76) and as the most consistent with the long-time average annual data of observations for Central Yakutia (HTI = 0.72).

The seeds were germinated in the containers filled at $^2/_3$ of the height with the sterile quartz sand with the particles size of 0.5-2.0 mm and 80% moisture capacity. The seeds were impressed into the sand with the tamper to the depth equal to their thickness and germinated in the dark at 20 °C. The germination energy was determined on the 4th day, the germination value – on the 8th day. The seeds' moisture was evaluated gravimetrically (MB45 device by Ohaus, Switzerland). The seeds of 10-g samples had been ground for 60 seconds using the rotary grinding system and had been dried for 20 minutes at 150 °C.

The values of seeds' germination and moisture of the initial samples, which have been determined before the beginning of the experiment, were 95-97% and 7% respectively. The accelerated seeds' aging was provoked according to the description [13]. The seeds were moistened in the thermostat at 37 °C and relative air humidity of 98%. The value of final seeds' moisture of each sample was 13.5%. The control of seed moisture was performed gravimetrically as described above. The comparative samples (controls) were the seeds which have not been subjected to additional moistening.

For biochemical and molecular studies the embryos tissues were used. The pea seeds were laid out in Petri dishes in a single layer and filled with distilled water at $^2/_3$; after the seed swelling for 12 hours the embryos were isolated.

The spectrophotometric measurements have been performed using the UV-2600 device (Shimadzu, Japan). The total content of low molecular weight antioxidants (LMWA) was determined according to the technique [15] based on the oxidation of antioxidants with iron chloride (III) with its reduction to iron chloride (II), the amount of which was measured basing on the change in staining intensity when the addition of o-phenanthroline (extinction coefficient $\varepsilon = 52.8\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 510$ nm). The peroxidase activity (POC, EC 1.11.1.7) was evaluated basing on the increase of the optical density due to the formation of the stained product of the o-dianisidine oxidation ($\varepsilon = 30\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 460$ nm) for 1 min [16]. The intensity of peroxidation of lipids (LPO) was evaluated basing on the accumulation of the stained complex of malondialdehyde (MDA) with thiobarbituric acid ($\varepsilon = 155\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 532$ nm) [17].

The DNA fragmentation degree in the isolated embryos was determined using the alkaline version of the DNA comet method (gel electrophoresis of single cells) with some modifications [18], which makes it possible to make the quantitative evaluation of the damages to DNA (single-stranded and double-stranded breaks, alkaline-labile purine and pyrimidine sites) [19]. After the period of seeds swelling, the isolated embryos were placed on ice in the Petri dishes of 60 mm diameter, coated with 250 μl of cold sodium phosphate buffer (pH 7.5), and then the incisions were accurately made on the embryos with the sharp razor blade. The dishes were kept in the ice in tilted position so that the nuclei, which become released from the embryos' cells into the buffer, accumulate in the dish lower part. The nuclei containing suspension was purified from major impurities using the nylon mesh filter with the holes size of 20 μm. Then 60 μl of the resulted suspension was placed in the test tubes with 240 μl of the 1% solution of low-melting agarose and applied on the glass slides previously coated with high-melting agarose. After the agarose solidification at the temperature of 4 °C, the micropreparations were placed in the alkaline buffer for electrophoresis (300 mM of NaOH, 1 mM of EDTA, pH > 13) for 20 min for causing the DNA denaturation and single stranded breaks in the alkaline-labile sites. The electrophoresis has been performed for 20 min at the field density of V = 1 V/cm and the current strength of ~ 300 mA, then the preparations were washed with sodium phosphate buffer (pH 7.5), fixed in the 70% ethanol solution and dried. Immediately prior to the microscopy (LabMed-2L fluorescence microscope,

Russia), the preparations have been stained with SYBR Green I fluorescent stainer (Sigma-Aldrich, USA; concentration 20 μg/ml) for 30 min and examined at ×200 magnification using the excitation and cutoff filters (490 and 530 nm respectively). The obtained images of DNA comets were analyzed using the CASP 1.2.2 software (http://casplab.com/download). The percentage of DNA in the comets' tail (the share of DNA in the comet tail in the total amount of DNA in the comet in %) has been used as the indicator of DNA damage degree. The atypical DNA comets with the absent or practically absent head and with a wide diffuse tail were put into the separate category and their number per 100 DNA comets was calculated [20].

All measurements have been performed in 4 replicates. The experiment results are presented in the form of arithmetic mean (*M*) and its standard error (±SEM). The samples were compared using the one-way analysis of variance (ANOVA), the statistical significance of the differences with control was determined using the Dunnett's test for multiple comparison at the significance level of $p < 0.05$. The calculation was made using the AnalystSoft package, StatPlus v. 2007 (AnlystSoft Inc., Germany).

*Results.* The brief description of the varieties and their hybrid used in the experiment is given in Table 1. The Melkosemyannyi 2 was originated in the Bashkir Research Institute of Agriculture (Ufa city) in 1961, and since 1963 it has been included in the State Register of Selection Achievements Authorized for Use. The Saryal variety was originated in 2002 in the Yakutsk Research Institute of Agriculture (Yakutsk city) by the method of individual selection for lodging resistance and early ripening on the base of an anonymous sample (USA), in 2015 it was transferred to the State Variety Testing, and since 2019 it has been included in the State Register of Selection Achievements Authorized for Use. The Saryal × Melkosemyannyi 2 hybrid was created in the same institute in 2004 and currently is undergoing the station tests in the breeding nurseries of the Yakutsk Research Institute of Agriculture.

**1. Studied forms of edible pea (*Pisum sativum* L.)**

| Variety, hybrid | Maturing rate | 1000 grains weight, g (*M*±SEM) | Seeds | | |
|---|---|---|---|---|---|
| | | | size | shape | color |
| Melkosemyannyi 2 | Mid early | 141±1.4 | Small | Spherical | White matte |
| Saryal | Mid early | 277±3.0 | Large | Round | White matte |
| Saryal × Melkosemyannyi 2 | Mid early | 201±2.0 | Medium | Rounded | White matte |

**2. Germination energy and germination rate of the edible pea (*Pisum sativum* L.) seeds depending on the period of exposure to the factors determining the accelerated aging (*M*±SEM)**

| Exposure, weeks | Germination energy, % | Germination rate, % |
|---|---|---|
| | Variety Saryal | |
| 0 (control) | 86±6 | 97±3 |
| 6 | 84±7 | 86±7 |
| 12 | 60±9* | 82±8* |
| 24 | 22±8* | 22±8* |
| | Variety Melkosemyannyi 2 | |
| 0 (control) | 78±8 | 96±4 |
| 6 | 48±10* | 94±5 |
| 12 | 30±9* | 46±10* |
| 24 | 8±3* | 8±5* |
| | Hybrid Saryal × Melkosemyannyi 2 | |
| 0 (control) | 62±9 | 95±4 |
| 6 | 74±9 | 99±1 |
| 12 | 66±7 | 84±7 |
| 24 | 26±8* | 32±9* |

* Differences with the control are statistically significant at $p < 0.05$ (ANOVA, Dunnett's criterion for multiple comparisons).

According to existing concepts, seeds aging conditioned by the oxidation processes in the seed dormancy period, which increases in the swelling period, leads to metabolic changes which, depending on the damage degree, is expressed in the slowdown of germination, reducing the viability and death of seeds [7].

The pea varieties and hybrid used in our researches initially (control) differed in the energy of seeds germination (Table 2). The Saryal variety had a high value (86%), the other two samples had a slightly lower values, 78% for Melkosemyannyi 2 and 62% for the hybrid. The germination of the investigated seeds was in the range of 95-97%.

In the course of accelerated aging after the 6-week exposure, the decrease in germination energy by 30% ($p < 0.05$) was noted only in the Melkosemyannyi 2 variety, that, however, have not affected the germination value which did not significantly differ from the control. In this variety, the exposure for 12 weeks led to the decrease in germination energy by another 18%, in germination value by 50% ($p < 0.05$). In the Saryal variety, the decrease in these indices relative to control (by 26 and 18%, respectively) also occurred. The significant changes in physiological parameters were observed after 24 weeks under conditions determining the artificial aging. In the Melkosemyannyi 2 variety the germination energy and germination value decreased to 8%, in the other two samples the germination energy was about 24%, in the Saryal variety the germination value decreased 4.4 times and in the hybrid 3.0 times as compared to the control. Thus, among the investigated samples, the seeds of the Melkosemyannyi 2 variety showed themselves as rapidly aging, the seeds of the Saryal variety had moderate resistance and the seeds of these varieties' hybrid showed the high resistance that may be conditioned by the heterotic effect. Our data on the variety-specific peculiarity of changing of physiological parameters in the course of seeds aging are consistent with the results of other researchers [21, 22].

During the storage, seeds are characterized by low moisture content which leads to their insignificant metabolic activity, while the autooxidation processes generate free-radical products of reactions [23]. Seed swelling is the most critical stage of seeds germination, which leads to the release of ROS (reactive oxygen species) formed both during the storage and due to the increased respiratory activity [7]. We have investigated the accumulation of MDA (malondialdehyde) (as the end product of lipids peroxidation) in the embryos' cells of the aging pea seeds after the 12-hour swelling (Fig. 1, A).

In the embryo cells of the seeds of two samples characterized by medium and high resistance to aging, a 1.5-fold increase in MDA accumulation as compared to the control (in the Saryal variety after 6 weeks, in the hybrid after 12 and 24 weeks) has been detected. In the Melkosemyannyi 2 variety, such increase has not been noted that (taking into account the highest aging rate in this sample) does not exclude the intense lipids peroxidation (LPO) in the period between 0 and 6 weeks. At the same time, after 24 weeks of exposure to aging factors, in this variety, the LPO intensity was 2.5 times higher than in the control.

It should be noted the absence of the linear dependence of LPO on the duration of accelerated aging.

It is deemed that low molecular weight antioxidants play a decisive role in the inactivation of ROS under the conditions of increasing oxidative stress that is conditioned, among other factors, also by the depletion of the pool and/or of the enzymatic antioxidants' activity in the course of long-time oxidative exposure [7, 24]. We have determined the LMWAs content (Fig. 2) and the peroxidase's activity (see Fig. 1, B) in the embryos' cells while the seeds' swelling at different time points during the artificial aging. Peroxidase is a bifunctional enzyme participating in peroxidation or oxidation and in the release of ROS. Also, this protein is related

with the cell elongation and growth limiting reactions [25, 26].

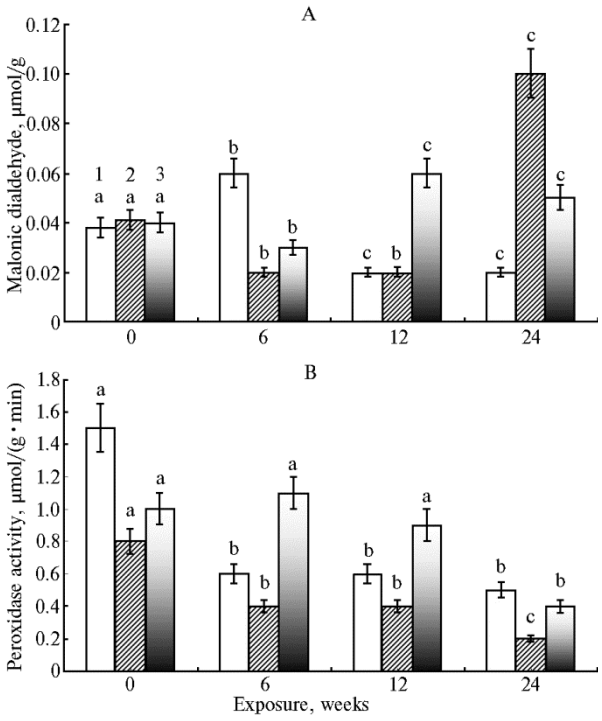


**Fig. 1. The accumulation of malondialdehyde** (MDA) (A) **and peroxidase activity** (POA) (B) **in the embryos' cells of the swollen seeds of pea (*Pisum sativum* L.) depending on the period of exposure to the factors determining the accelerated aging:** a — the control or the value indistinguishable from it; b — the value differing from the control; c — the value differing both from a and b (the differences are statistically significant at $p < 0.05$ according to the Dunnett's test for multiple comparisons).

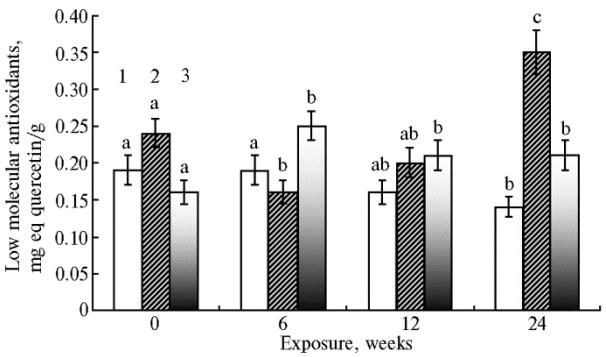


**Fig. 2. Content of low molecular weight antioxidants** (LMWA) **in the embryos' cells of the swollen seeds of pea (*Pisum sativum* L.) depending on the period of exposure to the factors determining the accelerated aging:** a — the control or the value indistinguishable from it; b — the value differing from the control; c — the value differing both from a and b (the differences are statistically significant at $p < 0.05$ according to the Dunnett's test for multiple comparisons).

The POA activity in the embryo cells of the rapidly aging seeds of the Melkosemyannyi 2 variety decreased as the exposure period extends, in 6-12 weeks 2 times, and after 24 weeks 4 times as compared to the control (see Fig. 1, B). Initially, LMWAs content decreased 1.5 times (6 weeks), and by the end of the 24$^{th}$ week it increased 1.5 times compared to the control (see Fig. 2). In the Saryal variety which showed the medium aging rate (among the investigated samples) the enzyme activity decreased by 60-65% for 6-24 weeks. At the same time, the LMWAs amount decreased by 23% ($p < 0.05$) only at the last term of accelerated aging. The hybrid, which was the most resistant, was characterized by the statistically significant 2.5-fold decrease of peroxidase activity (POA) only after 24 weeks of aging, while the LMWAs content was 1.4 times higher than in the control over the entire observation period.

Thus, the pea seed aging led to the decrease in peroxidase activity in the embryo cells of the swollen seeds. The change in the LMWAs content in the investigated pea samples under the conditions of increasing oxidative stress was multidirectional.

As the result of breach of the oxidation-reduction balance, which is conditioned by the excessive generation of ROS and decrease of the enzymatic activity, the breach of the DNA structure's integrity is initiated [7, 10]. Using the alkaline version of the DNA-comet method in our researches made it possible to make the quantitative comparison of the nuclear DNA fragmentation degree in the embryo cells in the swelling period be-

tween the control samples and the seeds subjected to accelerated aging (Table 3). In the control samples, the share of DNA in the comet's tail was 9-22%. The seed aging led to the statistically significant ($p < 0.05$) increase of the DNA damage degree compared with the control: in the Saryal variety by 13% since the 24th week of exposure, in the Melkosemyannyi 2 variety by 2.2-31.2% since the 12th week and in the hybrid by 3.5-20.5% since the 6 week. The comets with the absent or practically absent head and with a wide diffuse tail (the so-called "ghost cells" or "hedgehogs") have also been detected. The appearance of such atypical DNA comets is deemed an indicator of irreversible processes, i.e. cell death related to the strong oxidative stress, or of the formation of apoptotic cells being at the stage of chromatin fragmentation [27-29].

**3. The degree of damage to nuclear DNA in the embryo cells of pea *Pisum sativum* L. seeds depending on the period of exposure to the factors determining the accelerated aging** ($M \pm$SEM)

| Exposure, weeks | DNA in the comet's tail, % | Atypical DNA comets, % |
|---|---|---|
| | Variety Saryal | |
| 0 (control) | 22.3±1.8 | 1.0±0.3 |
| 6 | 18.0±2.5 | 4.7±0.5* |
| 12 | 20.7±2.9 | 10.5±0.5* |
| 24 | 35.3±0.8* | 17.0±1.7* |
| | Variety Melkosemyannyi 2 | |
| 0 (control) | 17.4±0.5 | 1.5±1.0 |
| 6 | 19.1±2.9 | 10.4±2.0* |
| 12 | 19.6±0.1* | 10.3±1.9* |
| 24 | 48.6±2.7* | 60.5±3.0* |
| | Hybrid Saryal × Melkosemyannyi 2 | |
| 0 (control) | 8.7±0.6 | 0.5±0.2 |
| 6 | 12.2±1.8* | 2.4*±0.6 |
| 12 | 13.1±1.3* | 7.5*±0.9 |
| 24 | 29.2±1.8* | 17.0±0.5* |

* Differences with the control are statistically significant at $p < 0.05$ (ANOVA, Dunnett's criterion for multiple comparisons).

The experiment showed the increase of the share of atypical comets in all the investigated samples of pea as the period of exposure to the factors of accelerated seeds aging extends. After the 24-week artificial aging, the number of atypical comets increased 17-40 times relative to the control depending on the variety. The comparison of the obtained data showed that when the atypical comet share increased to 10%, the seed germination energy decreased by 25-30% while the germination value either did not differ from the control or decreased slightly. This fact may testify about going of repair processes in the embryos' cells at the seeds' hatching stage and is consistent with the results of the researches [30, 31], in which it has been shown that under the effect of peroxide in the concentration which does not cause a cytotoxic effect, the repair of damaged DNA regions is possible. The further increase of DNA fragmentation led to the decrease of the seeds' germination energy and germination value, which testified about a certain critical level of damages to DNA and a significant decrease of the influence of DNA repair processes.

Thus, the results obtained in this work testify that the accelerated aging exerts different influence on the germination value and germination energy of the seeds, as well as on the biochemical characteristics of the embryo cells of the investigated varieties and inter-variety hybrid of edible pea. The Melkosemyannyi 2 variety showed the lowest resistance to the artificial aging conditions; the seeds of the Saryal variety showed medium resistance. The seeds of the inter-variety hybrid showed the highest resistance, which is probably owing to the heterosis effect. The influence of accelerated aging for 24 weeks led to the 1.6-3.3-fold increase of the DNA share in the comet tail while the number of atypical comets in different samples (varieties and hybrid) increased 17-40 times

compared to the control. The significant decrease in the physiological characteristics in the less resistant pea variety, which has been caused by the aging conditions, could be conditioned by the high degree of the nuclear DNA fragmentation, decrease of the antioxidant enzyme activities (in particular, of peroxidase), and by the increasing intensification of oxidative processes (exceeding the control value of lipids peroxidation by 2.5 times in the rapidly aging variety together with low germination) in the embryo cells. Based on the obtained data, it can be assumed that the increase of the DNA damage degree in the investigated pea varieties occurs due to the "depletion" of enzymatic antioxidant and repair systems and testifies about the slowdown or loss of restoration processes in the embryo cells of aging seeds.

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