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Effects of gibberellic acid and storage temperature on the germination of hawthorn seeds

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Abstract

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This study investigated methods to overcome seed dormancy in *Crataegus pseudoheterophylla* Pojarkova seeds. Seeds with and without endocarps were treated with gibberellic acid (GA_3) at different concentrations and four storage temperatures. Then they were stratified in an alternate temperature regime. The amount of absorbed water in seeds with endocarps was monitored by measuring the fresh weight of seeds for 0, 24, 48, 72, and 96 h of imbibition. The electrical conductivity (EC) and the percentage of water uptake by seeds stored for 12 months at laboratory temperature, in a refrigerator, in a freezer, and in freeze-thaw conditions were measured. The highest germination (59.7%) was recorded in seeds without endocarps treated with 3,000 $mg \cdot l^{-1}$ GA_3 and stored either in a laboratory or a refrigerator (32.7–35.3%). All treatments of seeds without endocarps where GA_3 was applied showed statistically higher percentages of germination than the control. Seeds with endocarps stored at refrigerator temperature imbibed water up to 44.3% with increasing imbibition periods, whereas the amount of seeds that absorbed water in freezer and freeze-thaw conditions was almost the same. The tests showed the highest EC during storage in the freezer, with the lowest water uptake and viability in seeds stored during the freeze-thaw process.

Keywords: *Crataegus* spp.; electrical conductivity; endocarp; seed dormancy; water uptake

Hawthorn (*Crataegus* spp.) belonging to the family Rosaceae is a large genus containing as many as 1,250 polymorphous wild species of shrubs and small trees. The distribution of hawthorn extends throughout western Asia including Afghanistan, Iran, Turkey, and the central Asian republics. Its edible fruit is small, rounded, and dark red in colour. Hawthorn propagation is usually through seeds. They have seeds with storage characteristics that can be dried to the low moisture content (9–13%) without considerable loss of viability. Seed germination is low because of double dormancy (physical dormancy and physiological dormancy) and it may take three years for seeds to germinate (Tipton, Pedroza 1986; Bujarska-Borkowska 2006). Germination in hawthorn seeds is related

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to fruit maturity, exogenous and endogenous dormancy, seed storage conditions, thickness of stony endocarp, and stratification period (Bujarska-Borkowska 2007). Removal of the endocarp may hasten or increase germination in stone species. Seed scientists have used various methods and technologies to break seed dormancy. Many studies have investigated the effect of exogenous growth regulators on overcoming the endogenous dormancy of seeds. The use of gibberellic acid (GA_3) has eliminated the chilling requirements of seeds and increased the percentage of germination – PG (Ghayyad et al. 2010).

Seeds having endogenous dormancy will not germinate until subjected to stratification (Hartmann et al. 2010). Several studies have been carried out on pre-germination treatments for *Crataegus* Linnaeus seeds. To release the seeds of *Crataegus monogyna* Jacquin from dormancy, a warm stratification is normally applied at 20°C for 4–5 months, followed by a cold stratification at 3–5°C for 4–5 months (Bujarska-Borkowska 2002). *C. monogyna* seeds with endocarps required 112 days of warm stratification followed by cold stratification to reach a PG of 75% (Bujarska-Borkowska 2002).

Using cold stratification alone (for 20, 40, 60, and 90 days) or 60 and 90 day of cold stratification with submersion in H_2SO_4 for different durations (30, 75, 105, 120, 150, and 180 min), and autumn sowing did not lead to the germination of *Crataegus microphylla* K. Koch, *C. monogyna*, *Crataegus pontica* K. Koch, and *Crataegus pseudoheterophylla* Pojarkova seeds. Germination occurred only in the *Crataegus monogyna* subsp. *azarella* (Grisebach) Franco seeds and the highest PG was 17.5% (Yahyaoglu et al. 2006). Mirzadeh Vaghefi et al. (2013) reported that soaking seeds without endocarps of *Crataegus aminii* Khatamsaz, *Crataegus babakhanloui* Khatamsaz, and *Crataegus persica* Pojarkova in 150 mg·l^{-1} GA_3 resulted in PGs of 28, 32 and 17%, respectively.

Many species produce seeds that do not germinate shortly after dispersal and that require a period of after-ripening during storage in different conditions (Gozlan, Gutterman 1999). Storage conditions and temperature are important factors in regulating the after-ripening process and breaking seed dormancy. Inappropriate storage temperature can often result in a low PG, cause seed deterioration, and lead to a loss of seed viability (Schmidt 2000). The effects of storage temperature and after-ripening on the permeability of the seed coat and then germination of *Haloxylon salicornicum* (Moquin-Tandon) von Bunge ex Boissier have been reported (Clor et al. 1976). In order to preserve the genetic resources in stored seeds, it is essential to maintain seed viability for longer periods (Murdoch, Ellis 2000). Storage temperature leads to changes in levels of growth inhibitors or stimulators within the seed coat and embryo (Bell 1999). Exposing seeds to temperature alternations between low and high is a method used to break seed dormancy (Rutar et al. 2001).

In order to determine the effect of storage temperature on seed germinability, it is essential to use the electrical conductivity (EC) test. EC is used to assess seed quality based on the permeability of the cell membrane system. The total concentration of electrolytes leached by seeds like ions, sugars, and other metabolites during soaking has long been indirectly assessed through the conductivity test (Son Mark et al. 1990).

There is an increasing interest in studies on seed dormancy and germination of *C. pseudoheterophylla* among nursery managers. This is so because the results can be directly applied to improve techniques for seed propagation. The present study aims to improve the germination of *C. pseudoheterophylla* stratified seeds by considering the effects of GA_3 treatment and storage temperature, and determine whether or not seeds have physical dormancy. Additionally, we evaluated the water uptake by seeds after 12 months of dry storage at different temperatures to determine whether seeds imbibe water. We also examined whether storage temperature affects seed vigour by measuring electrical conductivity and viability.

MATERIAL AND METHODS

Seed collection. Fully ripe fruits of the midland hawthorn (*C. pseudoheterophylla*) variety were collected from plant stocks of the Markazi province in Dokhaharan village, Shazand Arak, Iran (49°24'E, 33°51'N, 2,200 m a.s.l.) on October 30, 2011. In this region, mean annual rainfall, temperature, and humidity are 568 mm, 11.6°C and 50.5%, respectively. The drought period is approximately for 150 days, from June to the end of October. The climate is semi-humid, mountainous, and cold (Aghakhani, Metaji 2010).

Pulp extracts and determination of seed characteristics. Pulp was removed by wet maceration. After extracting the nutlets from fruits, the viability of seeds was examined using 2,3,5-triphenyl tetrazolium chloride (TTC) solution. Their moisture content was determined by the air-oven method (103°C, 17 h). Seed purity was computed by di-

viding the weight of pure seed by the total weight of the sample and then multiplying the amount by 100. The tests had four replicates of 25 nutlets (hereafter called seeds) each.

Experiments for improving germination. Seeds were immersed in running water for 48 h; afterwards they were placed on moist sand in perforated plastic boxes and kept in cold storage at –19°C for 24 h (Mirzadeh Vaghefi et al. 2010). Seeds were surface sterilized in a 2% NaOCl solution for 15 min and then rinsed with distilled water three times.

The seeds were subjected to warm stratification in a medium containing sand, perlite, and cocopeat (2:1:1), in a growth chamber for one month at a temperature of 23°C and with 50% moisture (Baskin, Baskin 2001). Next, the seeds were separated into two groups: those with endocarp and those without endocarp. To remove endocarps, seeds were placed one by one in a clamp and gently exposed to pressure by tightening the clamp. The clamp was tightened until a "click" sound was heard from the endocarp. The endocarp was then gently separated from the embryo without damaging the testa.

Treatments are described below:

(1) GA_3 (Sigma-Aldrich): Seeds with and without endocarps were imbibed in GA_3 solutions at concentrations of 0, 250, 500, 1,000, 1,500, 2,000, 2,500, and 3,000 mg·l^{-1} for 48 h at an ambient laboratory temperature. Then the seeds were stratified at an alternate regime: 1 month at 4°C, then 2 weeks at 23°C, followed by 1 month at 4°C, then 2 weeks at 23°C, and finally 1 month at 4°C (Ghayyad et al. 2010). All seeds were mixed in moist sand, perlite, and cocopeat (2:1:1) in polyethylene bags (8 cm diameter, 15 cm height). Seeds were aerated and checked for moisture at intervals of seven days. All treatments were repeated three times, with 100 seeds per repetition, in a factorial arrangement based on a completely randomized design. For this experiment, approximately 4,800 mature seeds were collected on the end date of harvest;

(2) Storage temperature: As seeds without endocarps would get spoiled during storage, they must be stored with endocarps. Four storage temperatures were tested: (*i*) dry-stored at laboratory temperature (22°C), (*ii*) in a refrigerator (4°C), (*iii*) in a freezer (–19°C), (*iv*) in a freeze-thaw cycle consisting of 7 days of exposure to –19°C and then 14 days of thawing at 22°C. Seventeen cycles were applied over a period of 12 months in moisture-proof containers. Stored seeds with endocarps were sterilized with the fungicide (0.2% carboxin thiram), and tests were done for viability (four replicates containing 50 seeds in each replication) by staining with 1% solution of TTC. The electrolyte amount released by soaking seeds in water was measured with an EC meter (Benchtop, 8,301 N, Hach Company, USA) used for measuring the EC of seeds stored at different temperatures; the mean values for each seed sample were expressed in μs·cm^{-1}·g^{-1} (Vieira et al. 2001). Then the seeds were removed from storage and divided into two parts (with and without endocarps) and stratified according to the previous regime (GA_3 method). All treatments were repeated three times, with 100 seeds per repetition, in a factorial arrangement based on a completely randomized design. For this experiment, approximately 2,400 mature seeds were collected on the end date of harvest.

Determining the percentage of water uptake. In 2012, the percentages of water uptake of seeds stored for 12 months at laboratory temperature, in a refrigerator, in a freezer, and in a freeze-thaw conditions were measured. The increase in the fresh weight of 50 seeds, with endocarps in three replicates for 0, 24, 48, 72, and 96 h of imbibition, at 4°C with deionized water was monitored (Eq. 1):

$$WS = \frac{(W_a - W_b)}{W_b} \times 100\,(\%) \quad (1)$$

where:

WS – increase in the mass of seeds,

W_a – mass of seeds after a given interval of imbibition,

W_b – initial mass of seeds.

Seed germination. After stratification, the polyethylene plastic bags containing seeds were transferred to a growth chamber with air circulation for germination. Seeds began to germinate 60 days after transferring the polyethylene plastic bags to the growth chamber. Then they were evaluated at intervals of three days for 90 days. Criterion of germination was the emergence of cotyledons above the soil mixture. The final PG was calculated when no further germination took place for seven days. Germination testing was terminated after 10 months. Afterwards, explants were transferred to polyethylene bags (8 cm diameter, 15 cm height) containing soil/cattle manure (2:1) for growing. The PG was computed by dividing the number of germinated seeds by total number of sown seeds and then multiplying the amount by 100.

Statistical data analysis. Distribution of data was tested for normality by the Kolmogorov-Smirnov test and all data were subjected to the arcsine ($\sqrt{x}$) transformation before statistical analysis. Homogeneity of variance among treatments was tested using Levene's test. A two-way ANOVA was performed on

the PG and differences between means were analysed by Duncan's multiple range test ($P = 0.05$) using SPSS (Version 17, 2008).

RESULTS AND DISCUSSION

Characteristics of seeds

The number of seeds per kilogram was 6,310, mean moisture content, viability, and purity of the fresh seeds used in the experiments were 10.2, 80.1, and 99%, respectively.

Effect of GA_3 on PG

Responses of seeds to GA_3 concentrations were significantly different in terms of statistics ($P < 0.000$). The highest PG for each seed group with endocarps (9%) and without endocarps (59.7%) was obtained at a concentration of 3,000 mg·l^{-1} GA_3. Seeds with endocarps showed the lowest germination at a concentration of 250 and 500 mg·l^{-1} GA_3 but the lowest germination in seeds without endocarps was obtained in the control (Table 1). The treatment with exogenous GA_3 stimulated the PG of all seed groups.

GA_3 increased germination in several species of the family Rosaceae (Ghayyad et al. 2010) and overcame physiological dormancy in seeds with dormant embryos (Hartmann et al. 2010). Seed dormancy may be caused by insufficient development of the embryo, chemical inhibition, or the failure of chemical reactions that make food reserves in the seed available to the developing embryo (Karam, Al-Salem 2001). Additionally, physiological dormancy in seeds is closely related to the proportion of the inhibitors, especially abscisic acid, and growth regulators, especially GA_3 (Hartmann et al. 2010).

Table 1. Percentage of germination of *Crataegus pseudoheterophylla* Pojarkova seeds with or without endocarp after different gibberellic acid (GA_3) treatments

GA_3 (mg·l^{-1})	Germination of seeds (%)	
	with endocarp	without endocarp
0	2.3 ± 0.3^{ef}	2.3 ± 0.3^{ij}
250	1.7 ± 0.3^{fgh}	8.3 ± 1.7^{fgh}
500	1.7 ± 0.3^{fgh}	10 ± 1.2%
1,000	2.3 ± 0.3^{ef}	22.3 ± 2.3^{de}
1,500	2.7 ± 0.3^{ef}	30.7 ± 4.3^{c}
2,000	4 ± 0.6^{cd}	50 ± 2.5^{b}
2,500	7.7 ± 1.2^{b}	58.7 ± 2.4^{a}
3,000	9 ± 0.6^{a}	59.7 ± 3.3^{a}

Values are given as means ± standard errors, all values with the same letters are not significantly different

For seeds with endocarps, the GA_3 treatment improved the germination, especially for seeds treated with 2,500 and 3,000 mg·l^{-1} at 9 and 7.7%, respectively, as compared to 2.3% germination for the control. This stimulating effect of GA_3 on seed germination can be attributed to a reduction of the preventive effect of the seed endocarp and its cytokinin activity in overcoming inhibition. Miransari and Smith (2014) reported that GA_3, through regulation of proteins, weakening of the endosperm, and expansion of the embryo cell, can release endocarp dormancy. A very important role of the endocarp is to provide mechanical resistance which can prevent the expansion of an embryo. The walls of the endocarp cells in the micropylar region are weakened by hydrolytic enzymes or a modification of the cell wall composition. However, the expansion potential of the embryo increases after GA_3 treatment, and warm and cold stratification. This helps the embryo to break the endocarp. Indeed, it has been suggested that the degradation of cell walls is required to allow for protrusion of the radicle from the seed (Bewley 1997).

The PG for seeds without endocarps treated with GA_3 was significantly higher than for seeds with endocarps. Removing endocarps increased overall germination as compared to seeds with endocarps (Table 1). Removing endocarps allowed the entrance of GA_3 into the seed, and promoted the production and activity of alpha-amylase enzyme (3-amylase) that transmutes starch into its simple sugar units, which are then consumed by the embryo. Also, removing endocarps reduces mechanical resistance of the embryo. The hardness of the seed endocarp, as an inhibiting factor in seed germination, has been studied in the seeds of several species of *Crataegus* (Yahyaoglu et al. 2006).

The effects of GA_3 on the releasing of seeds from physiological dormancy vary greatly, according to the deepness of dormancy (deep, intermediate, or non-deep). The germination of seeds with non-deep and intermediate physiological dormancy is increased with GA_3 treatments, whereas it does not promote the germination of seeds with deep physiological dormancy (Baskin, Baskin 1998). In the present study, seeds without endocarps and without exogenous GA_3 application showed a low germination rate and equal to the PG of seeds with endocarp. Also, there is little change in the PG with increasing GA_3 concentrations, reflecting that seeds have deep physiological dormancy.

The low germination of seeds with endocarps as compared to seeds without endocarps may be related to the hard stony endocarps or to the inhibitors around the seed showing its physical dormancy. In agreement with these results, Persson et al. (2006) demonstrated the inhibitory effect of the endocarp on the germination of *C. monogyna* seeds. Chemical pre-treatment of the seeds may be required to promote growth. The seed endocarp may act as an obstacle to light or as a barrier against the leaching of inhibitors from inside the seed. Also, the solubility of oxygen in water is low and phenolic compounds in the endocarp may use much of the oxygen for oxidation, thus restricting oxygen transport to the embryo (Persson et al. 2006). Results of our study confirm that the seeds of *C. pseudoheterophylla* have physical-physiological dormancy.

Effect of storage temperature on PG

After 12 months of dry storage at different temperatures, the highest PG for each seed group with and without endocarps was obtained from seeds stored in a refrigerator (4°C). Seeds with endocarp and without endocarp, stored in a refrigerator, had PGs of 22 and 35.3%, respectively (Table 2).

Research has shown that maintaining seed longevity depends on physiological factors as well as storage conditions (Schmidt 2000). The increase in emergence under dry storage conditions could be caused by rising temperatures due to the breaking of seed dormancy during storage in such conditions. Some reports have also demonstrated that dry storage increased the germination in many species including *Lonicera* sp. with underdeveloped embryo and *Prosopis juliflora* (Swartz) de Candolle with physical dormancy (Gutterman 2000; El-Keblawy, Al-Rawai 2006). Dry storage probably makes changes in the contents of growth inhibitors or growth promoters in the seed embryo that stimulate the seed to germinate (Baskin, Baskin 2001).

In the present study, seeds stored in a refrigerator germinated much better than those stored in a freezer (Table 2). In a refrigerator (4°C), both the temperature and the relative humidity were properly maintained, in order to maintain seed viability and germination capacity for a relatively longer period. Seed energy reserves are different between newly collected seeds and seeds subjected to long-term cold storage (Qian et al. 2009). Cold storage may reduce the endogenous abscisic acid levels within seeds, which would stimulate germination (Yang et al. 2008). Thomsen and Eriksen (2006) reported that in *Malus sargentii* Rehder and *Malus sieboldii* Rehder, among eight tested pretreatments of temperatures, only the seeds pretreated at a temperature of 4°C germinated. Higher PGs were recorded in Vaccinium ovalifolium Smith, Vaccinium deliciosum Piper, Vaccinium scoparium Leiberg ex Coville, Vaccinium caespitosum Michaux., and Vaccinium myrtillus Linnaeus after the seeds were dried and cold stored at 2°C for 6 to 12 months (Vander Kloet 1983). Seeds stored at 2°C retained higher viability (60.5%) than those stored at –10°C (30.0%) in *Populus ciliata* Wallich after one year of storage (Sah, Singh 1995).

Table 2. Percentage of germination of *Crataegus pseudoheterophylla* Pojarkova seeds with or without endocarp after storage at different temperatures

	Germination of seeds (%)	
	with endocarp	without endocarp
Laboratory (22°C)	20.3 ± 1.9[a]	32.7 ± 2.7[a]
Refrigerator (5°C)	22 ± 2.1[a]	35.3 ± 2.6[a]
Freezer (–19°C)	7.7 ± 1.3[c]	6 ± 1[c]
Freeze-thaw (7 days at –19°C, then 14 days at 22°C)	14.3 ± 2.1[b]	8.7 ± 0.9[c]

Values are given as means ± standard errors, all values with the same letters are not significantly different

After ripening, the proteins necessary for seed germination are accumulated; seeds continue the maturation process, and therefore food supplements in the embryo are optimal for the germination process (Miransari, Smith 2014). This could be due to the increasing PG during storage at 20 and 5°C in *Picrasma javanica* Blume (Setyowati 2009).

In our study, water uptake in imbibition periods at laboratory temperature, in a refrigerator, in a freezer, and in freeze-thaw conditions, from the beginning until the end of 96 h, was 41.6, 44.3, 35.2 and 29.4%, respectively (Fig. 1). After seed storage, water uptake in seeds stored in the freezer and in the freeze-thaw conditions was slower than in those stored at laboratory temperature and in a refrigerator (Fig. 1). This indicated that the embryo or other cells were damaged during storage. In our study, water uptake in the freezer and in the freeze-thaw conditions showed almost constant values in imbibition periods, while there was increased water uptake with an increase in the imbibition periods at laboratory temperature and in the refrigerator (Fig. 1).

The tests showed the highest EC during storage in a freezer (Fig. 2a). Similar results were obtained by Vieira et al. (2001) while studying the use of

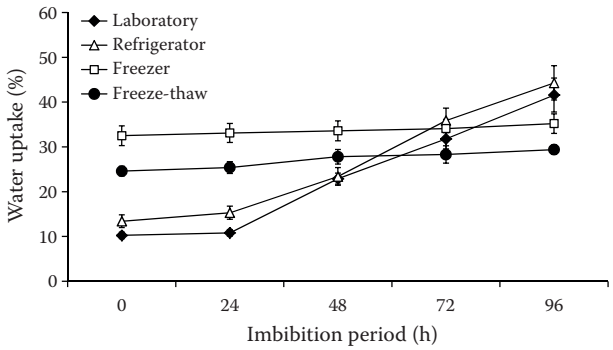

Fig. 1. Water uptake of *Crataegus pseudoheterophylla* Pojarkova seeds with endocarp after 12 months of dry storage at laboratory temperature, in a refrigerator, in a freezer and in freeze-thaw conditions. Initial water (moisture) content is plotted at 0 h. Vertical lines represent the standard errors of the means

soybean seeds in the EC test to determine seed vigour after storage at low temperatures. This may indicate that deterioration took place during storage in a freezer causing severe reductions in seed germination. The beginning of seed deterioration could be due to a gradual hydrolysis of soluble sugars. The hydrolysis of sugars present in seeds would lead to an accumulation of reducing sugars, which would finally threaten the proteins and cell membrane integrity (Sun, Leopold 1995).

In this study, viability levels of 80, 80, 40, and 58% were obtained in seeds stored at laboratory temperature, refrigerator, freezer, and freeze-thaw conditions, respectively (Fig. 2b). In the current study, seeds stored for 12 months at laboratory temperature and in a refrigerator exhibited similar viability as freshly collected seeds. Progressive loss of viability was observed in treatments in a freezer and the freeze-thaw cycle (Fig. 2b). Freezing plays a role in the breakdown of hard-seededness in species adapted to cold climates (Reed 2005). Lower germination in freezer conditions can be due to the compression of the seed endocarp by ice crystal formations, oxidation of food materials stored in the embryo, and loss of viability (Arbabian et al. 2009).

In general, seeds gradually release from dormancy during the storage period, and become permeable to water and gases. Water entering into the seed increases the seed moisture content, and thus reduces seed viability through increased respiration (Ruiz et al. 1999). However, the loss of viability could be due to simultaneous changes in vitamins, enzymes, respiration percentage, and the activity of endogenous hormones during storage (Ahmadi et al. 2001).

Freezing storage at –19°C and subsequent thawing had a negative effect on seed viability. Rutar et al. (2001) reported that the repeated freeze-thaw cycle seems to be an effective method of making a fragile seed coat. Transferring the seeds exposed to a low temperature to a higher temperature may act to recreate conditions that serve as a signal for the beginning of spring. Environmental conditions, particularly those produced by alternating temperatures, serve to regulate seed germination and dormancy by affecting the plant hormone balance of GA_3 and abscisic acid biosynthesis and catabolism, which will determine the dominant hormone (Cadman et al. 2006).

In this study, tests were done to remove dormancy in *C. pseudoheterophylla* seeds. Exogenous GA_3 (3,000 mg·l^{-1}) and storage in a refrigerator were successfully applied to seeds with and without endocarps, showing that seeds have a physical dormancy as evidenced by the very low PG of seeds with endocarps. Seeds with endocarps imbibed more water after 12 months of storage at refrigerator temperature, which shows that water penetrated into the seed coats during this time, began to soften, and subsequently initiated the PG. The highest EC, and the lowest water uptake and viability were obtained during storage in a freezer and the freeze-thaw process. This may indicate that during storage in the freezer and the freeze-thaw process,

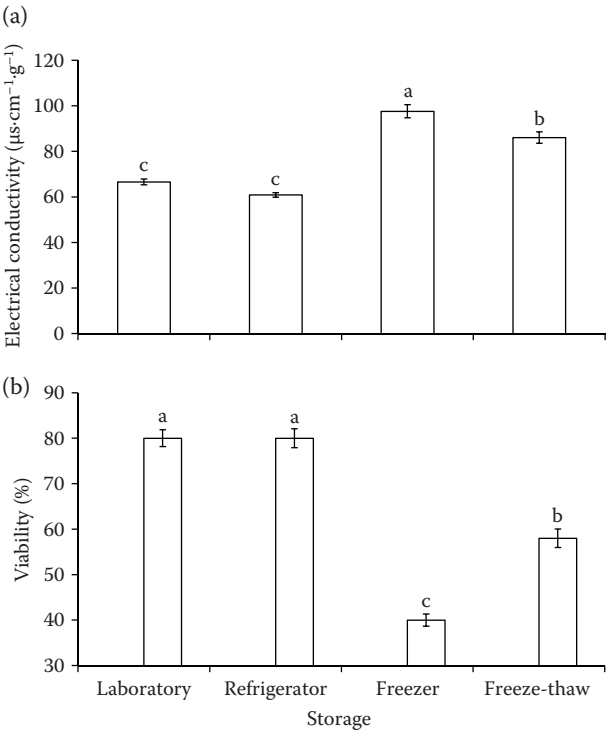

Fig. 2. Electrical conductivity (a), viability (b) of *Crataegus pseudoheterophylla* Pojarkova seeds with endocarp after 12 months of dry storage at laboratory temperature, in a refrigerator, in a freezer and in freeze-thaw conditions. Different letters on top of the columns are significantly different (one way ANOVA, Duncan's multiple range test, $P < 0.05$)

deterioration reached the point at which viability declines due to the higher amount of leachates into the imbibing solution.

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