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PHYSIOLOGICAL-BIOCHEMICAL PROPERTIES OF LEGUME SEEDS SUBJECTED TO LONG-TERM STORAGE

***Izabela Ćwiklińska*[1], *Martyna Dobak*[2], *Hanna Grajek*[3], *Dariusz J. Michalczyk*[4], *Wiesław Podyma*[5], *Agnieszka I. Piotrowicz-Cieślak*[6]**

[3] ORCID: 0000-0001-5439-3039
[4] ORCID: 0000-0002-3927-8806
[5] ORCID: 0000-0003-2018-281X
[6] ORCID: 0000-0001-8356-6373
[1,2,4,6] Department of Plant Physiology, Genetics and Biotechnology
[3] Department of Physics and Biophysics
University of Warmia and Mazury in Olsztyn, Poland
[5] Plant Breeding and Acclimatization Institute (IHAR)
National Research Institute in Radzików, Poland

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Abstract

Preservation of high physiological-biochemical seed quality is of paramount importance for seed storage. The effect of prolonged storage (30 years at the temperature +20ºC and -14ºC) of legume seeds (three cultivars of lupin and three cultivars of faba bean) was studied in this paper. The impact on seed vigour, viability, protein content and profile, and the activity of catalase and guaiacol peroxidase was analysed. Seeds stored at -14ºC germinated at 73.3% to 100%, whereas seeds stored at +20ºC completely lost germinability. Seeds of faba bean cultivars (*Vicia faba* subsp. *minor* 'Nadwiślański' and 'Stego'), and yellow lupin (*Lupinus albus* L. 'Iryd') had the highest viability. Since seed storage at +20ºC caused degradation of proteins with higher molecular weights, low molecular weight peptides predominated, in seeds subjected to such treatment, whereas seeds subjected to cold storage remained rich in high molecular weight proteins. Seed storage at room temperature also caused decreased catalase activity. The activity of this enzyme in seeds stored at -14ºC was 3.00 U, whereas in seeds stored at room temperature it dropped to 0.23 U.

Address: Agnieszka I. Piotrowicz-Cieślak, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-718 Olsztyn, Poland, phone: +48 (89) 523-42-89 e-mail: acieslak@uwm.edu.pl

Introduction

Seed storability depends on plant species. Seeds can be divided into orthodox, recalcitrant, and intermediate, depending on their tolerance to desiccation. Seeds that develop tolerance to drying and can withstand low water content (usually below 5%) retaining high vigour and viability are considered orthodox (PAMMENTER and BERJAK 2000). Such seeds acquire tolerance to desiccation by accumulating storage compounds (some carbohydrates and proteins) and adjusting cell metabolism – inhibiting respiration and modifying organelle differentiation (NIEDZWIEDZ-SIEGIEN et al. 2004). Recalcitrant seeds, on the other hand, reach maturity while they remain hydrated and they lose germinability if subjected to drying (WOJTYLA et al. 2006). The generally narrow life span of recalcitrant seeds makes their long-term storage a difficult task whereas, for orthodox seeds low water content and low temperature are known as the key factors favouring seed storability (RAJJOU et al. 2008). Despite generally good storability of orthodox seeds, they also eventually lose germination capacity as a result of long-term stora ge. The deterioration of seed quality during storage is a consequence of physiological-biochemical processes, e.g. changes in enzyme activities, decreased contents of carbohydrates, proteins and nucleic acids. The cell repair mechanisms are not efficient enough to make up for these changes (KAEWNAREE et al. 2011).

Previous studies of seed ageing have mostly used accelerated ageing tests (EKSI and DEMIR 2011, MATTHEWS et al. 2010, OHLSON et al. 2010, PROCHAZKOVA and BEZDECKOVA 2009, AL-MASKRI et al. 2003). Reports on long-term seed storage experiments and the impact of such treatments on seed viability are scarceVarious species of *Picea* seeds were analysed after 35 years of storage at −20ºC and were determined to have 60% viability (SIMPSON et al. 2004). In addition to the germination capacity, seed water content and the storage temperature were recorded (FOURAR-BELAIFA et al. 2011, RAO et al. 2006). Analyses of biogenic amine content and profile, proteins and soluble carbohydrates in legume seeds have also been carried out (DOBIESZ et al. 2017, DOBIESZ and PIOTROWICZ-CIEŚLAK 2017).

The objective of this paper was to determine the vigour and viability of legume seeds (lupin and faba bean) stored for 30 years at the temperatures of -14°C and +20°C. Moreover, the contents of proteins, their SDS-PAGE profiles and activity of catalase and guaiacol peroxidase in stored seeds were determined.

Materials and Methods

Biological materials

The experiments were carried out on seeds of two yellow lupin cultivars (*Lupinus luteus* L. 'Iryd' and 'Manru'), one white lupin cultivar (*Lupinus albus* L.'Hetman') and three faba bean cultivars (*Vicia faba* subsp. *minor* 'Stego', 'Nadwiślański' and 'Dino'. The experiments were started in 1988 and the seeds were stored for 30 years at -14°C and +20°C in air-tightly closed glass jars with a capacity of 1 l. The containers were filled with seeds to ¾ of their volume.

Seed vigour and viability

Seed vigour and viability were determined according to ISTA (2016) recommendations. To assess the viability, seeds were germinated for seven days on germination paper (Anchor Paper. USA) in a growth chamber (Sanyo incubator) at +20°C with 12-hour light provided by fluorescent tubes (840 lumens, Philips N.V., the Netherlands) and 12-hour darkness. Moreover, the fresh and dry mass of seedlings were determined after seven days of germination. The electroconductivity of seed leachates (exudates) was measured using a pH 211 meter (Hanna Instruments). For this purpose, the seeds were soaked for 24 hours in MQ water with initial electroconductivity of 0.03 μS.

Protein contents and SDS-PAGE separations

Proteins were isolated from defatted seeds (20 mg) in 0.5 ml tris(hydroxymethyl)aminomethane, pH 8, containing 0.01 M β-mercaptoethanol. The extraction was carried out for one hour and the extract was centrifuged at RCF 11 000 g and a temperature of 4°C. The total protein content of the extracts was determined with the Bradford method (Bradford 1976). The analysed protein fraction was dissolved in a buffer containing Tris-HCl (0.0625 M, pH 6.8), SDS (2%), glycerol (10%) and 2-mercaptoethanol (5%), to reach a final protein concentration of 2 mg ml^{-1}. The samples were heated for 5 min in a water bath at 100°C. They were then cooled and loaded onto a 10% polyacrylamide gel (7.0 cm × 10.0 cm) and subjected to SDS-PAGE in a Mini PROTEAN Tetra System (Bio-Rad). The separation was performed at 200 V for 40 min. After electrophoresis, the gels were stained with colloidal Coomassie Brilliant Blue G-250 (Sigma Aldrich). The gel images were digitized with a Gel Doc EZ Imager (Bio-Rad) scanner and analysed with ImageLab (Bio-Rad) software.

Guaiacol peroxidase activity assay

Seeds (100 mg) were homogenized with a porcelain pestle and mortar at 4ºC in 1 ml isolation buffer (0.01 M Tris-HCl (Sigma-Aldrich), 8.75% polyvinylpyrrolidone (Sigma-Aldrich), 0.1 M KCl (PPH Stanlab), 0.28% Triton X-100 (Sigma Aldrich)). The extract was centrifuged for 30 min at 2800 g, 4ºC. The protein content in the extracts was determined with the BRADFORD method (1976). The activity of guaiacol peroxidase was determined with a Cecil Aurius Series CE 2021 spectrophotometer (Cecil Instruments Ltd.). The extract (50 ul) and 25 ul of 0.06% H_2O_2 (Chempur) were added to 2 ml of the reaction mixture containing 0.1 M KH_2PO_4 (Chempur), and 100 µl 1% guaiacol (Sigma-Aldrich). The rate of absorption increase was measured at the wavelength λ = 470 nm at room temperature and the oxidation of 1 µmole H_2O_2 in one minute was assumed as one unit of peroxidase activity (RYDZYŃSKI et al. 2017).

Catalase activity assay

Seeds (100 mg) were homogenized in 1 ml phosphate buffer which contained 2% polyvinylpyrrolidone (Sigma-Aldrich), 0.02 mM EDTA (Sigma-Aldrich) and 10 ml l^{-1} Triton X-100 (Sigma-Aldrich). The extracts were centrifuged for 20 min at RCF 12 000 g, 4ºC. The protein content in the supernatant was determined with the BRADFORD (1976) method. Catalase activity was measured using a Cecil Aurius Series CE 2021 spectrophotometer (Cecil Instruments Ltd.). The reaction mixture contained phosphate buffer (50 mM, pH 7) and H_2O_2 (15 mM). Changes in absorbance at the 240 nm wavelength were followed for 10 min at room temperature and the oxidation of 1 µmole H_2O_2 in one minute was assumed as one unit of catalase.

Results and discussion

Seed viability and vigour

Seed age is an important factor strongly affecting germination capacity and the ability to produce good root and stem growth (BRUTOVSKÁ et al. 2013). Seeds that had been stored for 30 years at -14ºC germinated at the level 73.3% – 100%. The highest germination capacity was found for faba bean 'Nadwiślański', 'Stego', and yellow lupin 'Iryd' seeds. However, the lowest germination capacity (73.3%) was observed in white lupin 'Hetman'. Seeds stored at +20ºC did not germinate at all (Table 1, 2).

Table 1

Vigour and viability, germination [%], root and stem length [cm], seedlings fresh and dry mass [g], water content [%] and electroconductivity [μS g^{-1} fresh mass], of yellow lupin (*Lupinus luteus* L.) seeds 'Manru' and 'Iryd' and white lupin (*Lupinus albus* L.) seeds 'Hetman' stored for 30 years at + 20°C and -14°C

Specification		Lupinus luteus				Lupinus albus	
		'Manru'		'Iryd'		'Hetman'	
		+20°C	-14°C	+20°C	-14°C	+20°C	-14°C
Germination [%]		0	93.33±2.23	0	100	0	73.33
Length [cm]	root	0	8.571±2.742	0	7.733±2.868	0	11.512±6.046
	stem	0	4.514±1.148	0	2.887±1.712	0	4.195±1.897
Seedling fresh mass [g]	root	0	0.095±0.036	0	0.082±0.047	0	0.189±0.088
	stem	0	0.301±0.084	0	0.282±0.96	0	0.503±0.231
Seedling dry mass [g]	root	0	0.006±0.002	0	0.007±0.002	0	0.009±0.006
	stem	0	0.013±0.003	0	0.015±0.007	0	0.036±0.019
Water content [%]	root	0	92.81±1.734	0	88.981±3.315	0	95.501±2.128
	stem	0	95.327±0.793	0	94.585±2.694	0	92.786±2.079
Electroconductivity [μS g^{-1}]		217.01 ±23.7	184.3±4.1	165.16 ±4.81	30.41±2.7	201.31 ±3.83	56.81±2.57

Table 2

Vigour and viability viability, germination [%], root and stem length [cm], seedlings fresh and dry mass [g], water content [%] and electroconductivity [μS g^{-1} fresh mass], of faba bean (*Vicia faba* var. *minor*) seeds 'Stego', 'Nadwiślański' and 'Dino' stored for 30 years at +20°C and -14°C

Specification		*Vicia faba* var. *minor*					
		'Stego'		'Nadwiślański'		'Dino'	
		+20°C	-14°C	+20°C	-14°C	+20°C	-14°C
Germination [%]		0	93.333	0	100	0	100
Length [cm]	root	0	7.323±4.321	0	8.44±3.526	0	7.1±3.088
	stem	0	2.125±1.149	0	2.793±1.456	0	2.513±1.282
Seedling fresh mass [g]	root	0	0.205±0.091	0	0.28±0.077	0	0.239±0.043
	stem	0	0.205±0.101	0	0.24±0.099	0	0.215±0.088
Seedling dry mass [g]	root	0	0.011±0.007	0	0.032±0.017	0	0.019±0.009
	stem	0	0.007±0.005	0	0.017±0.006	0	0.016±0.007
Water content [%]	root	0	95.635±2.154	0	89.074±5.329	0	91.574±4.934
	stem	0	97.247±1.444	0	93.799±2.565	0	92.302±7.106
Electroconductivity [μS g^{-1}]		181.43 ±1.67	31.96±0.83	160.60 ±7.29	32.28±1.03	152.56 ±0.68	32.60±1.79

Seedling vigour assessments were based on mean root and shoot length. Mean root length (84.4 mm) was significantly higher than shoot length (31.7 mm). The longest roots (115 mm) were formed by white lupin 'Hetman' seedlings and the shortest roots (71 mm) by faba bean 'Dino'. The mean fresh mass of the roots (151 mg) was approx. twice smaller than the mean fresh mass of the stems (291 mg). Stems with the highest fresh mass were formed by white lupin 'Hetman' (503 mg), and roots with the lowest fresh mass (82 mg) developed in yellow lupin 'Iryd'. The highest dry mass of stem (36 mg) was recorded in white lupin 'Hetman', while the lowest dry mass of root (6 mg) was observed in yellow lupin 'Manru' seedlings. There were significant differences in water content across varieties (ALVES et al. 2017, CHATTERJEE and NAGARAJAN 2006, CHAUGHULE et al. 2005, OBROUCHEVA 2017, VERTUCCI and LEOPOLD, 1987).

The highest water content (97.2%) was observed in faba bean 'Stego' stems, while the lowest water content (89%) occurred in roots of yellow lupin 'Iryd'. Seed leachate electroconductivity was on average three times higher in seeds stored at +20°C, compared to those stored at -14°C. The mean exudate electroconductivity in seeds stored at +20°C was 179.67 $\mu S\ g^{-1}$, while in seeds stored at -14°C it was 59.41 $\mu S\ g^{-1}$. The highest exudate electroconductivity (217.01 $\mu S\ g^{-1}$) was measured in seeds of yellow lupin 'Manru', stored at +20°C, whereas the lowest electroconductivity (30.41 $\mu S\ g^{-1}$) was found in seeds of yellow lupin 'Iryd', stored at -14°C. The electroconductivity of leachates of faba bean 'Stego' seeds was nearly six times higher than in seeds of the same variety stored at -14°C. The lowest difference in electroconductivity between seeds stored at +20°C and those stored at -14°C (1.25 x drop) was observed in yellow lupin 'Manru'. Seed ageing under dry storage results from the gradual loss of integrity of cell membranes which are important barriers enabling undisturbed concurrent proceeding of various biochemical and physiological reactions. The impairments of cell membranes result in significant increases of their conductivity leading to the leakage of ions, amino acids and sugars (DEMIDCHIK et al. 2014, OUYANG et al. 2002).

One-dimensional electrophoresis

The patterns of polypeptide bands in fractions extracted from seeds stored at -14°C or +20 °C differed significantly in band numbers and intensities across the analysed cultivars. However, the differences between cultivars were small. Most polypeptides had similar molecular weights. Analysis of polypeptide bands in one dimensional electrophoresis was successfully applied to characterize protein profiles of different wheat cultivars. It

was found that protein profiles of stored seeds can be useful markers for studies of genetic variation and classification of cultivars increasing the efficiency of wheat breeding (SHUAIB et al. 2007). Using SDS-PAGE analyses VOIGT (1993) studied the presence of albumins and globulins in seeds.

The patterns of protein bands in SDS-PAGE were also analysed in seeds of various cultivars of chili peppers (*Capsicum* L.). The occurrence of 21 protein polypeptides was demonstrated with molecular masses 18.6 to 72.0 kDa (Figures 1, 2 and 3). The study revealed considerable differences between the genotypes. The variation of protein profiles suggested that selected genotypes could be good candidates for crop improvement by hybridization programs (KUMAR and TATA 2010).

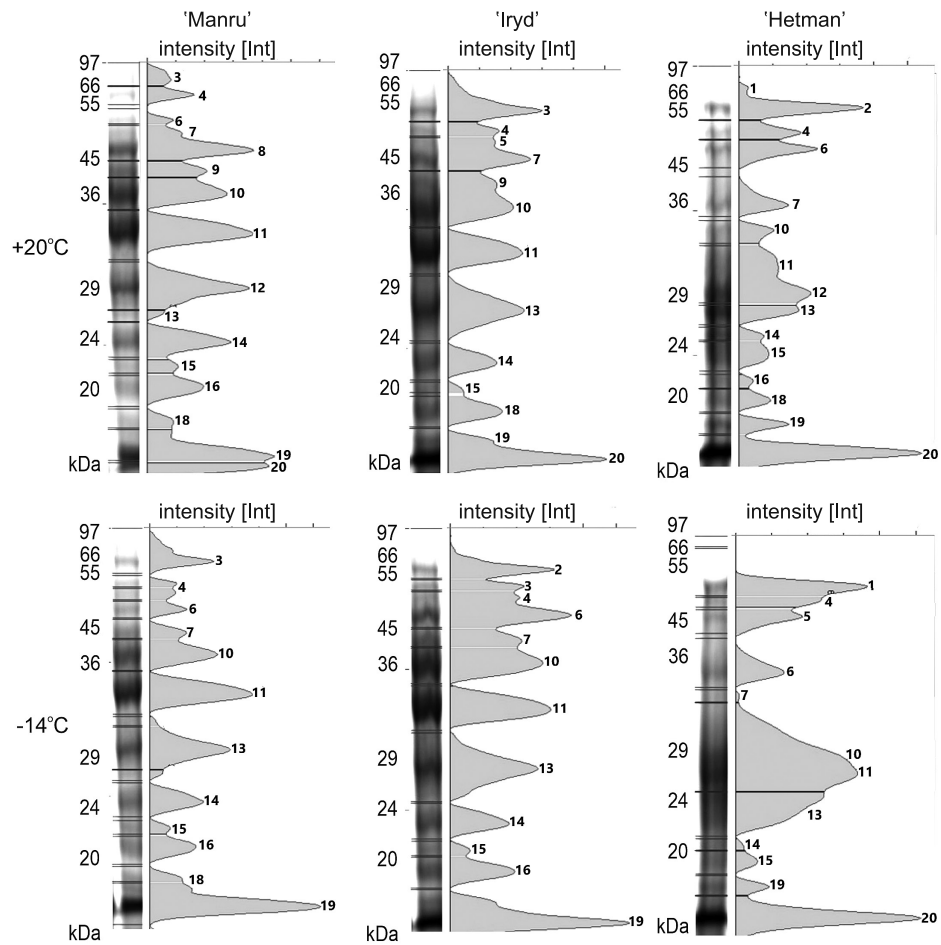

Fig. 1. SDS-PAGE electrophorograms and densitograms of lupin seed proteins. The seeds were stored at +20°C and –14°C during the 30-years period. Peptides sizes (kDa) and band numbers are given for each separation lane

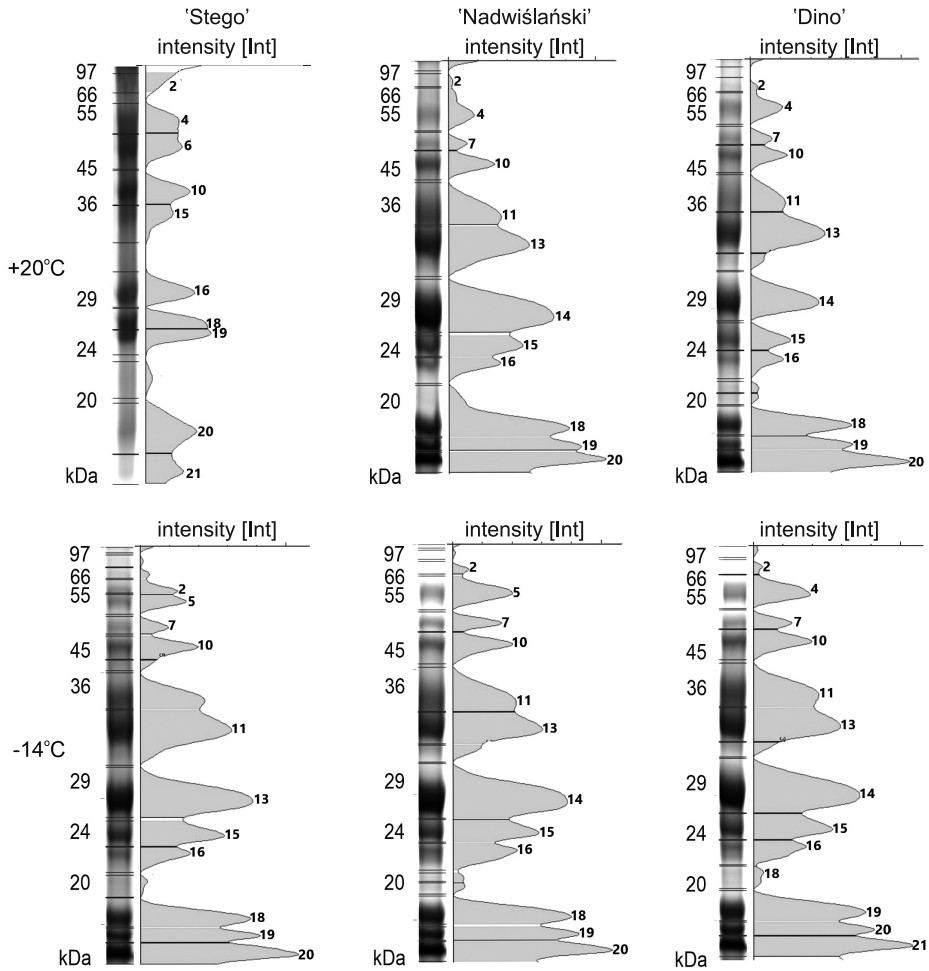

Fig. 2. SDS-PAGE electrophorograms and densitograms of faba bean seed proteins. The seeds were stored at +20°C and –14°C during the 30-years period. Peptides sizes (kDa) and band numbers are given for each separation lane

Seeds stored at -14°C were characterised by higher intensities of most polypeptide bands, compared to seeds stored at +20°C. This was particularly visible with bands of polypeptides with molecular masses 66 and 55 kDa (Figures 1 and 2). However, the intensities of bands corresponding to lower molecular mass polypeptides were higher in seeds stored at +20°C. The highest numbers of polypeptide bands were observed in seeds of yellow lupin ' Manru' and white lupin 'Hetman', stored at +20°C. Additionally, the band corresponding to a polypeptide with molecular mass 21.5 kDa was lacking in seeds of all studied cultivars of yellow lupin and white lupin

when the seeds were stored at -14 ºC (Figure 1). The faba bean seeds stored at +20ºC contained a protein with a similar but slightly higher mass 20.1 kDa (Figure 2).

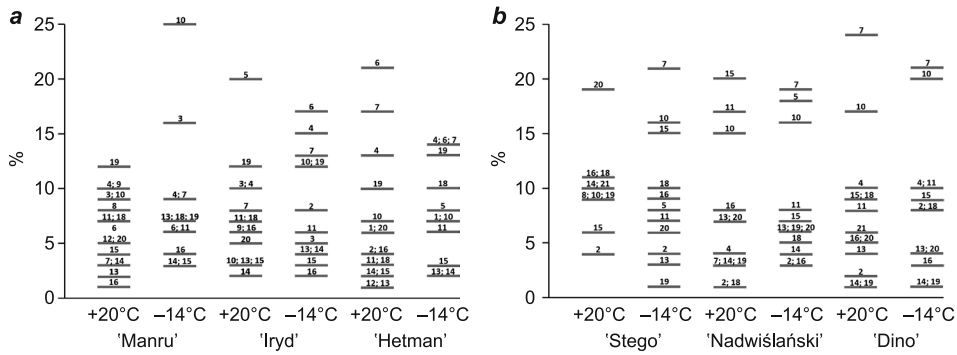

Fig. 3. Percent intensities of the polypeptide protein bands after SDS-PAGE separation of proteins. The numbers given above each band denote band location within each separation line (the lowest numbers correspond to the bands located closest to the sample wells); the percent values within each separation lane sum up to 100%. The seeds of lupin (*a*) and faba bean (*b*) were stored at different temperatures (+20 ºC, –14 ºC) during the 30-years period

Increased numbers and intensities of low molecular weight polypeptides in seeds stored at +20ºC suggest severe degradation of proteins during seed storage at such high temperature.

Enzyme activities

Seed enzyme activities differed depending on storage conditions. Temperature and ambient humidity are considered key factors in this regard (BALDOS et al. 2014).

Dehydration of plant tissues results in production of reactive oxygen species, which may interact with one another and start various detrimental oxidative reactions. The antioxidation defence is an important component of plant adaptation to desiccation, although it is not easy to precisely describe all its physiological contexts. The enzymes involved in antioxidation defence system show different susceptibility to seed ageing and are also affected plant species and seed storage conditions. The mechanisms of protection against reactive oxygen species involve free radicals, superoxide dismutase, catalase and the members of the ascorbate-glutathione cycle, such as glutathione reductase and antioxidative compounds, including reduced glutathione, ascorbate and α-tocopherol (BERNAL-LUGO and LEOPOLD 1992).

In seeds subjected to storage, changes in antioxidative enzyme activities were observed, e.g. catalase and peroxidases (AYYAPPAN et al. 2010). In drying seeds, catalases prevent damages resulting from dehydration. Hydrogen peroxide is probably involved in the regulation of catalase gene expression and signal transduction. In stored cucumber seeds a temporary increase of catalase activity was observed, which suggests the induction of antioxidative and anti-ageing protection system (AYYAPPAN et al. 2010). The decrease of catalase activity, observed later, was probably caused by insufficient biosynthesis of this enzyme as a result of seed ageing. Heat shock and oxidative stress contribute to catalase inactivation and prevent biosynthesis of new molecules of this enzyme (HERTWIG et al. 1992).

A significant decrease of peroxidase activity was observed in cucumber seeds subjected to accelerated ageing (AYYAPPAN et al. 2010). The decline of viability of stored wheat seeds is accompanied by decreases in activities of catalase, superoxide dismutase, and an increase in glutathione reductase activity (LEHNER et al. 2008).

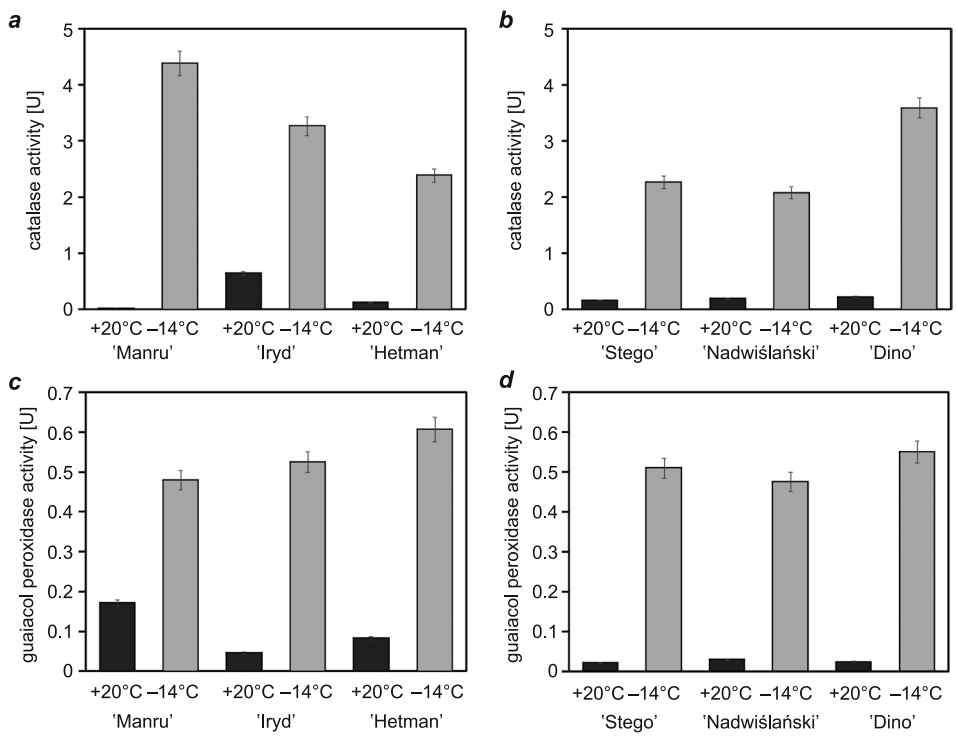

Fig. 4. Activity of catalase in lupin (*a*) and faba bean (*b*) seeds and guaiacol peroxidase activity in lubin (*c*) and faba bean (*d*) seeds (U one unit of enzyme activity corresponds to the oxidation of 1 µmole H_2O_2 for 1 min). The seeds were stored at +20 °C and –14 °C during the 30-years period

A few antioxidative enzymes of mung bean (*Vigna radiata*) – glutathione reductase, catalase and ascorbate peroxidase – were found highly sensitive to Maillard reaction (MURTY et al. 2003). In stored seeds, the Maillard reaction can result in chemical modifications of macromolecules, leading to a gradual decrease of seed ability to metabolically counteract the damages caused by free radicals during storage and germination. These changes cause decreasing seed viability and eventually seed death (MURTHY et al. 2003).

The decreases in enzyme activities can be caused by denaturation of the peptide part of the enzyme. It was shown in this paper, that a considerable decrease in enzyme activities occurs in seeds stored at +20°C in comparison to seeds stored at -14°C (Figure 4). Both the highest and lowest activities of catalase were noted in seeds of yellow lupin 'Manru'. In seeds stored at +20°C, the activity of this enzyme was 0.23 U, whereas in seeds stored at -14°C it was 3.00 U. The lowest activity of guaiacol peroxidase (0.021 U) was found in faba bean 'Stego' seeds stored at +20°C, while the highest activity of this enzyme (0.61 U) was detected in white lupin 'Hetman' seeds stored at -14°C. Mean activity of the guaiacol peroxidase in seeds stored at +20°C was 0.06 U and in seed stored at -14°C it was 0.525 U.

Conclusions

1. During 30 years of storage at -14°C the studied legume seeds retained high vigour and viability.
2. Seed storage at +20°C for 30 years resulted in degradation of high molecular weight proteins.
3. Activities of catalase and guaiacol peroxidase in seeds subjected to 30 years storage at +20°C were lower than in seeds stored for 30 years at -14°C.

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