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Physiological and Biochemical Parameters of Field Bean (*Vicia faba* var. minor) Seeds Stored for 33 Years

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Abstract: Changes occurring in seeds of two field bean cultivars during long-term storage at −14 °C, +4 °C and +20 °C were examined. It was found that after 33 years of storage at low temperatures, the seeds showed a significant decline in biological quality, and the seeds stored at +20 °C completely lost their germination capacity. As the seeds aged, changes in their phenolic composition, concentrations of polyamines and activity of enzymes associated with redox homeostasis and phenolic metabolism could be easily observed. The activity of ascorbate peroxidase and phenylalanine ammonia-lyase in deteriorated seeds was higher than in high-quality seeds. The activity of guaiacol peroxidase, catalase and glutathione reductase in low-quality seeds was decreased. With superoxide dismutase, the pattern was less clear-cut and depended on both seed biological quality and cultivar. Whole seed levels of spermine and spermidine decreased in ageing seeds and were lowest in non-germinating seeds. However, the opposite pattern was observed regarding spermine and spermidine, as well as putrescine, in seed coats. The obtained results indicate that changes in the activity of redox and protective systems in seeds have a clear relationship with the seed biological quality and can be detected even at a moderate level of seed deterioration. The analyses of such changes can significantly facilitate the assessment of seed quality and can therefore be of interest for seed companies and seed banks.

Keywords: *Vicia faba* var. minor seeds; long-term storage; peroxidase; superoxide dismutase; glutathione reductase; phenylalanine ammonia-lyase; biogenic polyamines; tannins; procyanidins

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1. Introduction

In recent years, we have been witnessing the rapid expansion of the global human population alongside the contraction of arable land availability and accelerating climate changes [1–3]. Simultaneously, agricultural production methods have undergone unification and intensification. The combined action of these factors makes it increasingly important to maintain the genetic diversity of crop plants and to preserve germplasm for future breeding projects. This key challenge can primarily be tackled by the maintenance of germplasm collections, especially in seed banks. The possibility of long-term storage in seed banks, while maintaining a high viability of seeds, can significantly reduce the cost of maintaining such priceless collections.

According to the standard international recommendations, field bean seeds should be stored in airtight containers within the temperature range from −20 °C to −15 °C and with low relative humidity [4]. The gradual deterioration of seeds resulting from ageing, however, occurs even under the appropriate storage conditions. The first visible signs of ageing include a decrease in their germination capacity and the appearance of abnormal seedlings [5]. Although seed ageing is the subject of intensive research, the mechanisms

of this process are still not fully understood [6]. The damage of cellular components by oxidative stress can significantly limit the longevity of seeds, particularly stored at low humidity. It is believed that reactive oxygen species (ROS) are responsible for the ageing process [6] and reduced antioxidant potential of cells [7]. The accumulation of ROS in seeds results in oxidative stress and can lead to damage in nucleic acids, lipid peroxidation in membranes, inhibition of protein synthesis or destruction of protein structures, disturbances in mitochondria, activation of programmed cell death and loss of activity of repair systems switched on by seed rehydration. Consequently, seeds gradually lose the ability to germinate [8,9]. Studies on ageing mechanisms in seeds subjected to long-term storage still require diverse investigations, as ageing is a complex process involving changes at the genetic, proteomic and metabolic level. For instance, the link between the degradation of storage proteins in seeds and reduced vigour and viability is not obvious and was rarely the subject of detailed studies; however, the previous work of our team [10] demonstrated such a relation really exists.

Studies of seed quality deterioration can elucidate in detail the causes and mechanisms of this multifaceted degradation process, potentially offering insights into the ways to diagnose, delay or even partially reverse this process [9]. Furthermore, markers that can be conveniently used to assess the advancement of the ageing process in seeds are still being sought [8]. Seed coats are the most visible components of seeds and are the tissues most directly affected by the environment. The low permeability of the seed coats can be an important factor, among others, contributing to the longevity of seeds, among others, by limiting damage caused by oxidative stress [11,12]. The seed coats contain numerous secondary metabolites such as anthocyanins, isoflavones and polyphenols. The main role of these substances in plants seems to be to protect plants against pathogens [13] but, in seeds, these metabolites participate both in inhibiting the development of microorganisms as well as removing ROS, thus maintaining the longevity of seeds [14]. In Arabidopsis seed coats, soluble proanthocyanidins are converted into unstable quinone molecules that tend to form brown polymers (condensed tannins) through the action of the TRANSPARENT TESTA TT10 gene product (AtLAC15) [15]. The levels of phenolic compounds and biogenic polyamine contents in seed coats could be applied as sensitive and non-destructive markers of ageing in seeds subjected to long-term storage. Piotrowicz-Cieślak et al. [10] showed that the deterioration of the quality of seeds stored in inappropriate conditions may result from the oxidation or condensation of phenols present in seed coats.

Biogenic amines are a particularly interesting group of metabolites due to their common occurrence and clear association with the regulation of plant growth and responses to stress [16,17]. There is a wide range of very common polyamines—di-, tri-, tetra-, penta- and hexa-amines—found in all living organisms. The main polyamines, the most common, are diamine putrescine (Put) and tetraamine spermine (Spm). However, some polyamines occur rarely or even sporadically [18]. The mode of action of biogenic amines is due to their cationic properties (amine and imine groups are strongly protonated [19] at physiological pH (weak bases), which results in a very dynamic mechanism of regulation). However, this aspect requires further research.

In ageing seeds, the antioxidant system undergoes important changes. Decreases in the activity of antioxidant enzymes—guaiacol peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD)—have been reported in seeds subjected to natural (slow) ageing [20]. Oxygen and hydrogen peroxide are known to be involved in the catabolism of polyamines, which are ubiquitous reducing and protective compounds of pro- and eukaryotes. In fact, there are some experimental data indicating the effects of seed ageing on seed level of polyamines, and, conversely, the effects of polyamines on the germination performance of ageing seeds, e.g., in onion [21], hot pepper [22] and sorghum [9]. Another aspect of seed deterioration that is rarely considered in studies of seed physiology is the activity of phenylalanine ammonia-lyase (PAL). However, it was shown in transgenic *Arabidopsis* that the overexpression of PAL resulting from the introduction of exogenous soybean PAL gene promotes seed vigour under high-temperature and -humidity stress

(conditions of accelerated ageing) [23]. In this paper, we analysed the effect of the long-term storage of field bean seed under conditions of low to moderate temperature and low humidity on the activity of PAL.

Biochemical–physiological parameters, if sensitive enough, could be used to assess the degree of ageing of seeds stored for a long time. Therefore, the aim of this study was to assess the vigour and viability of seeds of two cultivars of field bean *Vicia faba* L. (var. minor) stored for 33 years at temperatures of −14 °C, +4 °C and +20 °C. For this purpose, measurements of the content of phenolic compounds in seed coats were carried out and the levels of polyamines in seed coats and the whole seeds were determined. Furthermore, activities of phenylalanine ammonia-lyase and antioxidative enzymes were evaluated.

2. Materials and Methods

2.1. Material

The experiments were carried out using field bean seeds (cv. Nadwiślański and Dino) obtained from Plant Breeding and Acclimatization Institute—National Research Institute in Radzików, Poland. The seeds (lots with masses of approx. 1 kg) were stored for 33 years in tightly closed twist glass jars at −14 °C and +4 °C and in linen bags at +20 °C (in temperature-controlled cabinets). The relative air humidity in containers with the analysed seeds was determined the day before the analysis and was 52% ± 4, 49% ± 2 and 46% ± 2, respectively, for the storage temperature of −14 °C, +4 °C and +20 °C. The measurements were carried out in five repetitions using a hair hygrometer model TR 415 (C.P. Polska).

2.2. Methods

2.2.1. Seed Quality

After 33 years of seed storage (1989–2022), their biological quality was determined. Seeds (10 in 5 replicates) were evenly placed on a damp sprouting paper (Anchor paper, St Paul, MN, USA). Germination was carried out at a temperature of +20 °C ± 1. Seeds were considered viable if after 7 days the radicle had penetrated the seed coat.

The average root and stem length and the dry and fresh weight of the seedlings were determined after seven days.

2.2.2. Isolation of Total Phenols

The seed coats were mechanically removed from the seeds of both cultivars, stored at +20 °C, −14 °C and +4 °C. Seed coats (200 mg) were ground in an electric grinder. Total phenols were isolated by placing 200 mg of ground seed coats in 30 mL of 70% acetone and shaking at 100 RPM for 48 h. The extracts were centrifuged at 3000× *g* for 10 min at 4 °C. A total of 4 mL of the supernatant was collected and dried in a vacuum oven, then dissolved in 4 mL of methanol and analysed.

2.2.3. Determination of Total Phenols, Non-Tannin Phenolics and Tannins

The determinations were performed using the Folin–Ciocalteu method in accordance with the Joint FAO protocol [24]. Folin's reagent (250 µL; diluted 1:1 with distilled water) and 1.25 mL of 20% calcium carbonate were added and incubated for 40 min in the dark. The absorbance at λ = 725 nm was then measured. The content of total phenols was calculated from the standard curve prepared for tannic acid. Phenols non-tannin phenolics were determined by adding 100 mg of insoluble PVPP (Merck, Poland) and 1 mL of water to 1 mL of the extract. The samples were incubated for 15 min at 4 °C, vortexed and then centrifuged at 3000× *g* for 10 min at 4 °C. The supernatant was collected. The content of non-tannin phenols was determined according to the Joint FAO protocol [24].

2.2.4. Determination of Polyamines in Seed Coats and Whole Seeds

Biogenic amines were extracted from seeds or isolated seed coats using cold 5% perchloric acid. The powdered material was shaken with 25 mL of 5% $HClO_4$ solution for 30 min and then centrifuged at 16,000× *g* for 30 min at a temperature of 4 °C. The

supernatant was evaporated, and the residue was dissolved again in 3 mL of 5% $HClO_4$. The extract was analysed using an AA 400 amino acid analyser (Czech Republic). Polyamines were separated at a temperature of 70 °C using a cation-exchange column measuring 7.0 × 0.37 cm, filled with Ostion Lg ANB. Biogenic amines were eluted using two citrate buffers at pH 5.65, adding 1.0 M HCl and 2.6 M sodium chloride. The quality and quantity of biogenic amines were determined using a spectrophotometric detector after reacting with ninhydrin.

2.2.5. Determination of Catalase Activity

Catalase activity was determined according to Orzoł and Piotrowicz-Cieślak [25] with modifications. Embryos (500 mg), i.e., seeds with seed coats removed, were homogenised in a cold mortar on ice in phosphate buffer pH 7 with 0.2 mM EDTA, 10 mL L^{-1} Triton X-100 and 10 g L^{-1} PVP. Samples were centrifuged at 12,000× *g* at 4 °C for 20 min. The supernatant was collected and the protein content was determined by the Bradford method [26]. Catalase activity was determined spectrophotometrically in a reaction mixture composed of 50 mM phosphate buffer (pH 7) and 15 mM H_2O_2 and 20 µL of enzyme in a total volume of 3.00 mL. Absorbance was measured for 5 min at room temperature at λ = 240 nm. Catalase activity unit was 1 µmol oxidised H_2O_2/min/mL.

2.2.6. Determination of Guaiacol Peroxidase Activity

Embryos (500 mg) of seeds were homogenised in a cold mortar on ice for 30 min in 0.1 M Tris-HCl, pH 5.5. With the addition of 8.75% PVP (Merck, Warszawa, Poland), 0.1 M KCl, 0.28% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The extracts were centrifuged at 4000× *g* at 4 °C for 30 min. The supernatant was collected and the protein content was determined by the Bradford method [26]. Guaiacol peroxidase activity was assessed by spectrophotometry (CECIL, CE2021 2000 Series) in a reaction mixture composed of: 100 µL 1% guaiacol (Sigma-Aldrich), 2 mL 0.1 M KH_2PO_4 (Chempur, Piekary Śląskie, Poland), 50 µL supernatant and 20 µL 0.06% H_2O_2 (Chempur) in a total volume of 3.00 mL. Absorbance was measured for 10 min at room temperature at λ = 470 nm. Guaiacol peroxidase activity unit was assumed to be 1 µmol of oxidised H_2O_2/min/mL.

2.2.7. Determination of Superoxide Dismutase Activity

The assay was carried out following the method of Goel et al. [20] with a buffer (pH 7.8) containing 200 mg PVP, 10 mmol/L 2-mercaptoethanol, 10 mmol/L KCl, 1 mmol/L $MgCl_2$ and 1 mmol/L EDTA. The homogenate was centrifuged twice at 15,000× *g* for 15 min at +4 °C. Protein content was measured by the Bradford method [26]. The reaction mixture contained 3 mL of 0.1 mol/L phosphate buffer (pH 7.8) containing 1.3 mol/L riboflavin, 13 mmol/L methionine, 63 mol/L nitroblue tetrazolium and 0.1 mL enzyme extract in a total volume of 2.00 mL. Glass tubes containing the mixture were exposed to light (50 µE m^{-2} s^{-1}). Unilluminated test tubes were used as a control. After irradiation for 10 min, the absorbance at 560 nm was measured. One SOD unit was defined as the level of enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50% (50% inhibition = 1 unit of SOD).

2.2.8. Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's comparison post hoc test ($p \leq 0.05$) was applied to evaluate differences between the storage variants: (seeds stored at temperatures −14 °C, +4 °C and +20 °C) using software Statistica 12.0 (StatSoft, Krakow, Poland). At least five replicates were used for all measurements.

3. Results and Discussion

3.1. Seed Quality

In this paper, we started by re-examining the performance of field bean seeds cultivars Nadwiślański and Dino after 30 years of storage (reported previously—[10]) and compared

those results to the data from recent experiments, carried out after an additional three years of storage of subsamples of the same seeds. Furthermore, we added additional analyses (activities of oxidative-stress-related enzymes, and phenylalanine ammonia-lyase, as an enzyme essential for various plant stress responses and for the biosynthesis of phenolic compounds). We report the changes in the seed contents of biogenic polyamines, levels of phenolic compounds and colouration of seed coats. As it was shown in our previous work that seeds stored for 30 years at +20 °C completely lost their germination capacity, we added in our current analyses seeds with an intermediate level of deterioration, i.e., stored for 33 years at +4 °C. Seeds stored at −14 °C exhibited a significantly lighter seed coat colour, ranging from light brown. In contrast, seeds stored at +4 °C displayed a dark brown seed coat (some of these seeds were even close to black in colour). Finally, seeds stored at +20 °C appeared predominantly black (see Supplementary Material Figure S1).

The light brown or beige colour of the seed coat is typical of field bean seeds after harvest. Robertson and El-Sherbeeny [27] indicate that as many as 91% of seeds are characterised by this colour of the outer cover; however, the authors emphasise that the colour is unstable and seeds get darker during storage. In addition, it has been shown that storing field bean seeds at different temperatures for 12 months leads to a significant reduction in the total amount of phenolic compounds, especially in the seed coats, which is associated with seed darkening [28].

In our experiments, field bean seeds that had been stored at −14 °C for 33 years exhibited germination rates of 91% and 77% for cv. Nadwiślański and Dino, respectively. These rates represent a further decline from the germination capacity levels measured after 30 years of storage as reported in our previous paper (98 and 91% for cv. Nadwiślański and Dino, respectively [10]). Therefore, it is evident that even under relatively optimal storage conditions, seed quality parameters visibly deteriorate between 30 and 33 years of storage in both field bean cultivars. However, this effect is more pronounced in the case of cultivar Dino. Seeds of both cultivars stored at +20 °C did not germinate at all (Tables 1 and 2). Seeds stored for 33 years at +4 °C exhibited relatively poor germination and seedling growth parameters. Seedling growth was assessed based on the average root and stem length. The average root length was 112 mm and 104 mm, respectively, for field bean seeds of the Nadwiślański and Dino cultivars stored at −14 °C. The stems of faba bean plants were notably shorter, measuring 29 mm and 26 mm for the Nadwiślański and Dino cultivars, respectively.

Table 1. Germination of field bean seeds cv. Nadwiślański [%], fresh weight [mg] and dry weight [%] of seedlings grown from seeds stored for 33 years at different temperatures. Means (±SD n = 10 to 15) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test).

	Storage Temperature		
	−14 °C	**+4 °C**	**+20 °C**
Water content in seeds, %	7.1 ± 0.08 A	6.3 ± 0.06 AB	4.5 ± 0.46 B
Germination, %	91 ± 2 A	27 ± 4 B	0 C
Length, mm root	112.4 ± 9 A	94 ± 7 A	0 B
stem	28.9 ± 24 A	0 ± 0 B	0 B
Seedling fresh weight, mg	1321 ± 46 A	1268 ± 26 A	0 B
Seedling dry weight, %	11.2 ± 2.4 A	11.3 ± 1.5 A	0 B

3.2. The Contents of Phenolic Compounds

Analysis of the composition of phenolic compounds in extracts from seed coats of field bean cv. Nadwiślański and Dino showed that seeds stored at +4 °C and +20 °C contained fewer phenolic compounds than those from −14 °C. It should be noted that the content of phenolic compounds in the seed coats of field bean cv. Nadwiślański and Dino was similar. Seeds stored at −14 °C contained almost twice as many phenolic compounds compared to those stored at +20 °C. Higher contents of non-tannin phenols and total

phenols were also found (Table 3). There were similar trends in the content of non-tannin phenols, their content being highest in seeds stored at −14 °C and lowest in seeds stored at +20 °C. Flavan-4-ols, free gallic acid and gallotannin were found to be lowest in stored seeds (Table 3).

Table 2. Germination of field bean seeds cv. Dino [%], dry matter] and fresh [mg] and dry weight [%] of seedlings growing from seeds stored for 33 years at different temperatures. Means (±SD, n = 10 to 15) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test).

	Storage Temperature		
	−14 °C	**+4 °C**	**+20 °C**
Water content in seeds, %	7.5 ± 0.1 A	6.1 ± 0.4 AB	4.5 ± 0.2 B
Germination, %	77 ± 7 A	50 ± 1 B	0 C
Length, mm root	104.3 ± 5 A	46.2 ± 4 B	0 C
Stem	26.3 ± 8 A	0 ± 0 B	0 B
Seedling fresh weight, mg	1116 ± 27 A	647 ± 34 A	0 C
Dry weight of seedlings, %	11.0 ± 2.4 A	11.6 ± 1.5 A	0 B

Table 3. Contents of phenolic compounds in seeds of field bean varieties Nadwiślański and Dino stored for 33 years at +20 °C, +4 °C and −14 °C. Means (±SD, n = 5) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test.

	Storage Temperature					
	−14 °C		**+4 °C**		**+20 °C**	
	Nadwiślański	**Dino**	**Nadwiślański**	**Dino**	**Nadwiślański**	**Dino**
Total phenols, mg tannic acid × 100 mg^{-1} ± SD	1.25 ± 0.017 A	1.26 ± 0.07 A	1.27 ± 0.02 A	1.28 ± 0.05 A	0.66 ± 0.05 B	0.79 ± 0.04 B
Non-tannin phenols, mg tannic acid × 100 mg^{-1} ± SD	0.505 ±0.001 A	0.475 ± 0.003 A	0.495 ± 0.015 A	0.580 ± 0.03 A	0.211 ±0.03 B	0.206 ± 0.03 B
Total tannins, mg tannic acid × 100 mg^{-1} ± SD	0.745 ± 0.017 A	0.788 ± 0.080 A	0.721 ± 0.040 A	0.697 ±0.050 A	0.454 ± 0.030 B	0.589 ± 0.020 AB
Flavan-4-ols, mg × 100 mg^{-1} ± SD	0.055 ± 0.008 A	0.047 ±0.009 A	0.061 ±0.005 A	0.063 ±0.009 A	0.066 ± 0.004 A	0.068 ± 0.008 A
Free gallic acid, mg × 100 mg^{-1} ± SD	0.045 ± 0.006 A	0.046 ± 0.009 A	0.049 ±0.003 A	0.048 ±0.008 A	0.053 ±0.005 A	0.054 ±0.002 A
Gallotannin, mg × 100 mg^{-1} ± SD	0.039 ± 0.004 A	0.036 ± 0.003 A	0.041 ±0.005 A	0.042 ±0.007 A	0.043 ±0.004 A	0.046 ±0.006 A

3.3. The Contents of Biogenic Polyamines

Phenolamides, also described as hydroxycinnamic acid amides, are products of the conjugation of phenolic acids and polyamines and may be the main forms of phenolic metabolites in seeds [29]. Polyamines, like phenols, are products of the conversion of amino acids and can undergo rapid transport in plant cells and serve as signalling and protective molecules [30]. Although their relevance for seed ageing has not been studied as intensely as in the case of membrane lipids, ROS or protein glycation products, there are some reports showing that polyamines can be indicators of seed ageing and in some cases can even be used to alleviate the effects of this deteriorative process, when applied at the stage of seed priming (gentle rehydration, not yet allowing germination but leading to seed metabolic activation [31]). Our experiments confirmed the presence of spermine, spermidine and putrescine in all seed lots of field bean as well as cadaverine in some of the seed lots. When the extracts of whole seeds were analysed, spermine and spermidine were the dominating species of PA, and their contents decreased with decreasing seed quality (Table 4). Surprisingly however, the opposite trend was noticed when the contents of PA were analysed in seed coats rather than the whole seeds (Table 5). Here, we could see that

putrescine was even more abundant than spermine and spermidine and the levels of all these PA increased with declining seed quality.

Table 4. Contents of biogenic polyamines in whole seeds of field bean varieties Nadwiślański and Dino stored for 33 years at +20 °C, +4 °C and −14 °C. Means (pmol/mg fw whole seed ± SD, $n = 5$) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test.

	Storage Temperature		
	−14 °C	**+4 °C**	**+20 °C**
		Nadwiślański	
Spermine	216 ± 12 A	134 ± 11 B	49 ± 9 C
Spermidine	257 ± 23 A	167 ± 27 B	27 ± 4 C
Putrescine	68 ± 6 A	70 ± 18 A	50 ± 11 B
Cadaverine	11 ± 2	tr	0
		Dino	
Spermine	324 ± 26 A	136 ± 3 B	97 ± 3 C
Spermidine	235 ± 12 A	157 ± 4 B	102 ± 9 C
Putrescine	84 ± 4 A	85 ± 6 A	90 ± 8 A
Cadaverine	31 ± 3 A	17 ± 4 B	0

Table 5. Contents of biogenic polyamines in seed coats of field bean varieties Nadwiślański and Dino stored for 33 years at +20 °C, +4 °C and −14 °C. Means (pmol/mg fw seed coat ± SD $n = 5$) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test; tr—trace amounts.

	Storage Temperature		
	−14 °C	**+4 °C**	**+20 °C**
		Nadwiślański	
Spermine	32 ± 2 A	56 ± 5 B	87 ± 3 C
Spermidine	47 ± 5 A	63 ± 4 B	112 ± 5 C
Putrescine	84 ± 4 A	170 ± 8 B	310 ± 7 C
Cadaverine	tr	8 ± 2 A	21 ± 4 B
		Dino	
Spermine	27 ± 3 A	48 ± 3 B	69 ± 3 C
Spermidine	39 ± 4 A	64 ± 6 B	96 ± 6 C
Putrescine	69 ± 4 A	98 ± 5 B	260 ± 5 C
Cadaverine	0	0	0

It is an interesting observation because seed coats seem to be metabolically rather inert, particularly in dry seeds; still, they reflect the physiological state of seeds quite clearly. Furthermore, they can be isolated from seeds and analysed without sacrificing the embryos, so this approach allows for non-destructive seed analyses. The results of whole seed analyses for PA contents in field bean, reported here, might seem to disagree with the pattern of PA levels in lupin seeds reported in our previous paper [32] (in that case, the PA levels increased with a decline in seed vigour). This discrepancy could probably be explained by the well-established fact that lupin seeds have exceptionally thick seed coats (with a mass over 25% of seed mass) [32]. In such seeds, the seed coat contribution to the overall seed metabolite pool can be significant. Interestingly, some researchers describe PA as juvenility factors, particularly in plants; however, a similar pattern of PA declining with age was observed in yeasts and animals [33].

3.4. Enzyme Activity

The "free radical theory of ageing" is well established in the literature and it assumes an important role of ROS in the progression of the time-related deterioration of cells and

organisms as well as aetiology of various age-related disorders. Antioxidative systems, both non-enzymatic (α-tocopherol, i.e., vitamin E, ascorbic acid, i.e., vitamin C, polyphenolics, flavonoids and carotenes) and enzymatic, including Fe-SOD in chloroplasts and Cu–Zn SOD in the cytosol, chloroplasts and possibly the extracellular space, as well as inner mitochondrial enzymes of the ascorbic acid—glutathione cycle, including Mn-SOD, APX, CAT, GR, GSH or others—are crucial elements of cell homeostasis [34–39]. These enzymes become activated in response to various stresses, e.g., drought [40,41] and environmental toxins [25,42]. Seeds, in order to preserve vigour and produce normal seedlings, need to balance ROS generation and scavenging. Seed viability often strongly correlates with the activity of the antioxidant system [39].

Among the enzymes studied in this paper, superoxide dismutase (the method applied did not allow for distinguishing the specific isoforms) and glutathione reductase had the lowest activity and L-phenylalanine ammonia-lyase was most active. A similar range of activity was shown by catalase, ascorbate peroxidase (APX) and guaiacol peroxidase (GPX; Table 6). Ascorbate peroxidase (APX) catalyses the decomposition of hydrogen peroxide using ascorbate as an electron donor, leading to the formation of monodehydroascorbate. During storage, the activity of APX in seeds stored at +20 °C reached a value that was about twice higher than that detected in seeds stored at −14 °C. In seeds stored at 4 °C, an intermediate level of activity was observed. APX is considered one of the most sensitive enzymatic indicators of oxidative stress. Similar levels of GPX activity were detected in seeds stored at −14 °C and +4 °C; however, the level of this enzyme activity decreased by approximately 50% in seeds stored at +20 °C. GPX removes H_2O_2 with concomitant oxidation of phenolic metabolites. Considering the changes in both total phenolic compounds and GPX activity, it seems probable that GPX is strongly involved in detoxication reactions in seeds with a low or moderate level of deterioration and low intensity of seed coat coloration. Seeds with black seed coats are characterised by low viability, low activity of GPX and low content of total phenols. A similar pattern of changes was visible in the catalase response to seed ageing. Glutathione reductase (GR) is responsible for maintaining the appropriate level of reduced glutathione (GSH) in the cell and reduced GSH controls the cellular levels of reactive oxygen species. We observed a comparable decrease in GR activity in seeds stored at +4 °C and +20 °C. Our data show that storage affects hydrogen peroxide metabolism in seed coats from the early stage of seed deterioration.

PAL activity controls the accumulation of phenols (including primary metabolites like phenylalanine and tyrosine, as well as secondary compounds, including flavonoids and diverse phenylpropanoids). It is not surprising, therefore, that the activity of this enzyme in seed coats was very high. Phenylalanine ammonia-lyase commonly occurs in plants, catalysing the conversion of phenylalanine to cinnamic acid with the release of ammonia. PAL activity correlates with the content of some fractions of the phenol metabolites (Table 6) [43]. In seed coats from seeds stored at +20 °C, an almost three-fold increase in enzyme activity was observed. It can be assumed that the key compound involved in the darkening of seed coats is a metabolite of phenylalanine. Phenylalanine is a key precursor of flavonoids and procyanidins. Procyanidin, also known as a condensed tannin, is a metabolite responsible for the darkening of the seed coat [44,45]. This compound is an oligomer or polymer of flavan-3-ols, which are formed in the flavonoid biosynthesis pathway. Oxidative reactions can lead to the degradation of procyanidins, which play a crucial role in protecting seeds from oxidative damage caused by various environmental factors like light, oxygen, high temperature, free radicals and metal ions. Procyanidins act as powerful antioxidants, reducing agents, scavengers of free radicals and chelators of pro-oxidant catalytic metals. Compared to simple phenolics, condensed tannins exhibit a significantly higher capacity (15–30 times more effective) to neutralize peroxyl radicals, thus preventing lipid oxidation [46].

Table 6. Enzyme activity (catalase, guaiacol peroxidase, superoxide dismutase, ascorbate peroxidase, glutathione reductase, L-phenylalanine ammonia-lyase) in seeds of field bean varieties Nadwiślański and Dino stored for 33 years at +20 °C, +4 °C and −14 °C. Means (±SD, $n = 5$) sharing the same letter in a row are not significantly different ($p \leq 0.05$) according to Tukey's HSD test.

	Storage Temperature					
	−14 °C		+4 °C		+20 °C	
	Nadwiślański	**Dino**	**Nadwiślański**	**Dino**	**Nadwiślański**	**Dino**
Catalase activity (μmol H_2O_2/min/mg protein ± SD	2.320 ± 0.150 A	1.730 ± 0.120 B	2.200 ± 0.070 A	1.780 ± 0.100 B	1.370 ± 0.180 C	1.350 ± 0.090 C
Guaiacol peroxidase activity, μmol H_2O_2/min/mg protein ± SD)	1.690 ± 0.207 A	1.690 ± 0.137 A	1.410 ± 0.260 B	1.690 ± 0.116 A	0.806 ±0.086 C	0.700 ± 0.071 C
Superoxide dismutase activity (50% inhibition = unit of SOD/mg fresh weight ±SD	0.123 ± 0.003 A	0.110 ± 0.014 A	0.246 ± 0.036 B	0.135 ± 0.033 A	0.056 ± 0.005 C	0.171 ± 0.038 C
Ascorbate peroxidase 1 μmol $H_2O_2 \times min^{-1}$)	1.3 ± 0.1 A	1.4 ± 0.3 A	1.8 ± 0.3 B	1.7 ± 0.1 B	2.4 ± 0.2 C	2.6 ± 0.1 C
Glutathione reductase (1 μM NADPH × min^{-1})	0.01 ± 0.002 A	0.02 ± 0.001 A	0.006 ± 0.0001 B	0.007 ± 0.0006 B	0.007 ± 0.0006 B	0.008 ± 0.0004 B
L-phenylalanine ammonia-lyase (μmol trans-cinnamic acid × mg^{-1} protein × 2 h^{-1})	80 ± 2.1 A	74 ± 1.6 A	143 ± 5.3 B	152 ± 7.4 B	210 ± 6.9 C	220 ± 7.2 C

The current study represents a continuation of research conducted by our team for over 30 years on the same varieties of field bean (cv. Nadwiślański and Dino). In our previous work [10], we investigated the ageing process of seeds at two distinct temperatures (−14 °C and +20 °C), in which significantly different dynamics of degenerative changes were observed. Under experimental conditions, we analysed protein composition (using 2D electrophoresis), seed coat pigmentation and phenolic content in relation to seed viability indicators. In the present study, we provide the results of analyses conducted on seeds of the same varieties but subjected to storage at three different temperatures (−14 °C, +4 °C and +20 °C) for the period of thirty-three years. Furthermore, in this study, we examined the activity of enzymes responsible for oxidative stress and the metabolism of phenolic compounds. We found that among the enzymes studied, ascorbate peroxidase and phenylalanine ammonia-lyase were the most sensitive indicators of a decline in seed vigour. Similarly, the contents of polyamines in whole seeds and seed coats can indicate changes in stored seed quality. Furthermore, the degree of seed vigour reduction in the tested varieties could be easily assessed based on the pigmentation of their seed coats.

However, it should be noted that *Vicia faba* is a plant with high genetic and physiological variability, with a wide array of features resulting form long-term breeding efforts by humans rather than biological determinants (various forms of *Vicia faba*, including var. minor and major, differ in plant and seed sizes, and can readily crossbreed [47]). Significant variation also exists in seed pigmentation. Therefore, for a practical application of the observations presented here, it would be necessary to calibrate the method for specific field bean cultivars. 'Nadwiślański' and 'Dino' are cultivars that, at the time of establishing the collection used in this study, were among the most commonly cultivated and extensively researched in Poland, as well as highly regarded for their high yield [48]. Both of these varieties produce lightly colored seeds.

Detecting changes in aged seeds is crucial for ensuring the quality and viability of seeds used for planting, which is essential for farmers and seed companies. Over time, seed viability can decline, resulting in low germination rates and reduced crop productivity. Seed banks, often maintained by specialised institutes, national (like National Centre for Plant Genetic Resources in Poland) or global (like the Svalbard Global Seed Vault), store

seeds for extended periods, often spanning decades. Therefore, detecting quality changes in these seeds is vital to guarantee the long-term conservation of plant genetic resources. Few experimental data are available on seeds subjected to long-term storage. A fairly extensive paper of this type was published on various crops seeds from the seed bank of Bulgaria [49]. This paper focusses on field bean seeds but provides more physiological-biochemical assessment criteria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13102012/s1, Figure S1: The appearance of field bean seeds cv. Dino: A—seeds stored at −14 °C, B—seeds stored at +4 °C, C—seeds stored at +20 °C and cv. Nadwiślański: D—seeds stored at −14 °C, E—seeds stored at +4 °C, F—seeds stored at +20 °C.

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