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Activity of α-D-Galactosidase in Long-Stored Seeds of *Vicia hirsuta*

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Abstract: Mature seeds of many crop species contain substantial amounts of soluble carbohydrates, including raffinose family oligosaccharides (RFOs) and cyclitol galactosides (Gal-C). These substances are hydrolysed by α-D-galactosidase (EC 3.2.1.22) during the early stages of germination, providing metabolic energy for the germination process. A decrease or inhibition of α-D-galactosidase activity can significantly reduce the germination rate. This study aimed to investigate changes in α-D-galactosidase activity during the long-term storage of *Vicia hirsuta* seeds and evaluate the association between the loss of enzyme activity and the decline in seed vigour and viability. *V. hirsuta* seeds were stored at 22 °C and 35% relative humidity for up to 25 years, and α-D-galactosidase activity and seed vigour changes were monitored at different time points. Storage of *V. hirsuta* seeds resulted in a loss of viability and vigour, accompanied by a decrease in α-D-galactosidase activity. The enzyme activity showed a significant correlation with seed germinability and vigour. Monitoring α-D-galactosidase activity in seeds subjected to long-term storage could be a simple and rapid method for determining the decline in vigour in seeds that utilize soluble galactosides as their storage materials.

Keywords: ageing; seed storage; seed vigour; galactosidase

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1. Introduction

Seeds are adapted to transport in space and time. They are an important source of food and feed, a starting material for crop production and a convenient form of plant genetic resources. During storage, in particular under unfavourable conditions such as high temperature and humidity, seeds deteriorate, lose vigour, become more sensitive to stresses during germination and their germinability decreases [1–4]. Long-stored seed are, therefore, characterised by reduced vigour. According to ISTA [5] seed vigour is the sum of properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments. Seed vigour is complex physiological trait related to rate and uniformity of seed germination and seedling growth, and the ability to establish a normal seedling even under unfavourable environmental conditions and maintain the ability to germinate after storage [6]. Seed vigour is important for successful establishment a new plant in the field and high crop yield. The decreased seed vigour during storage reduces seed sowing quality and usefulness. Efficient agricultural production largely depends upon preservation of viability and longevity of high-quality seeds during storage [7].

Changes accompanying the decay of longevity and vigour during seed storage for different time have been reported for many decades, and seed storability was determined for many economically important species [8–11]. Furthermore, seed longevity greatly varies not only between species, but also within the species [12–15]. The previous studies have also led to developing procedures for testing and quantifying changes of stored seeds quality. There is no universal seed vigour testing method; however, an array of methods can be applied (the cold test; the accelerated ageing test; controlled deterioration; tests based on seedling growth and biochemical tests, such as the electrical conductivity test

and the tetrazolium test) [16,17]. The most obvious manifestation of the onset of seed ageing is the decline in germination rate, followed by a decrease in seedling size, and an increased incidence of abnormal seedlings [17]. Thus, in practice, seed vigour is assessed commonly by both seed germination and seedling growth tests [18]. Among tests based on seedling performance are the first count of the germination test, rate of germination or seedling emergence, germination uniformity, and seedling growth (length or dry weight) tests. Based on the germination-time curve data estimated by using the four-parameter Hill function [19,20], it is possible to determine biologically relevant parameters of stored seeds, such as time to reach 50% or 75% germination (t_{50}, t_{75}), germination rate ($1/t_{50}$ or $1/t_{75}$), and uniformity of germination (for example U_{8020}: the interval between time points when 20% and 80% of seeds germinate). Germination rates (i.e., speed and distribution in time) reveal information about timing, uniformity and extent of germination in seed populations, and are sensitive indicators of seed vigour and stress tolerance [21]. The above parameters reflect changes in quality of a seed population. The time required for germination increases as seeds deteriorate during storage [22,23], and values of the other seed quality parameters change as well. Values derived from standard germination and seedling growth parameters, i.e., length and dry weight, are used to calculate seed vigour indexes [24].

Many physiological, transcriptomic, proteomic and metabolomic studies have been performed to identify factors contributing to the long term preservation of seed viability and determining the dynamics of seed ageing [23,25–37]. A few reviews papers summarizing the current state of knowledge of the mechanisms of seed longevity have recently been published [6,7,38–42]. The publications show that protection, detoxification and repair as well as hormonal signaling are the main systems allowing seeds to survive in the dry state, cope with age-related damages and stay able to germinate. However, quality and viability of seed during storage depends not only upon the initial status of the seed health, but also on the environmental factors operating during seed storage, including air temperature, relative humidity, and oxygen partial pressure [37].

Seeds of many economically important crops and wild-growing plant species contain soluble α-D-galactosides, that comprise more than 10% of their mass. These includes the widespread galactosides of sucrose—Raffinose Family Oligosaccharides (RFOs) and less common galactosides of cyclitos, also known as Galactosyl Cyclitols (Gal-C) [43–48]. Both, RFOs and Gal-C accumulate during seed development and maturation, and the increase in their content coincides with desiccation acquisition and with an increase in total germination percentage [45,49–51].

Seed galactosides are soluble low-molecular weight oligosaccharides that are a readily available energy source, mobilized at early stages of germination. RFOs and Gal-C are mostly degraded before the mobilization of the main storage materials begins, providing carbon skeletons and energy for the high-energy-demanding metabolic processes occurring in germinating seeds [52]. Hydrolysis of galactosides is required for rapid seed germination [53]. The bulk of galactosides is degraded even before germination is complete [18,54]. Hydrolysis of these sugars is a consequence of the increase of cells hydration and changes in pH inside cells. During germination, vacuoles are acidified by proton pumps [55], which promotes activity of acid α-D-galactosidases (EC 3.2.1.22, α-D-galactoside galactohydrolase), key enzymes responsible for the degradation of soluble galactosides in germinating seeds. α-D-galactosidases catalyse the hydrolysis of α-(1→6)-linked terminal D-galactose moiety from RFO and Gal-C molecules. Free galactose is converted rapidly to D-galactose-1-phosphate by galactokinase and further metabolized by using either the conventional Leloir pathway involving UDP-D glucose-hexose-1-phosphate uridyltransferase, or a pyrophosphorylase pathway involving UDP-D galactose pyrophosphorylase [56]. Galactose released from galactosides, in addition to providing easily available energy, may also be an important component of the signaling pathway during germination [53,57].

Seeds of legume species contain multiple isoforms of α-D-galactosidase that differ in optimum pH, molecular mass and structure, amino acid sequence, substrate specificity and expression pattern during seed development and germination [58–60]. Proteins with α-D-galactosidase activity are expressed and accumulate in protein storage vacuoles already during the seed development. High activity of galactosidase was found in the developing and mature dry seeds of many species [58,61–64]. It is assumed that the enzymes necessary for the resumption of metabolic activity during the initial germination phase are generally already present in mature, dry seeds. Presumably, an acidic isoform of galactosidase, pre-existing in mature seeds, is responsible for the rapid breakdown of RFOs and Gal-C in the initial stages of germination [48,58,65]. At later germination stages, alkaline galactosidases are synthesised *de novo*. In pea seeds, acidic α-D-galactosidases are involved in the rapid breakdown of RFOs during imbibition and germination, while the complete degradation of RFOs at early stages of seedling development involves the activity of a newly expressed alkaline α-D-galactosidase activity [58]. In plant species, the α-D-galactosidase expressed during germination, together with (1→4)-β-mannan endohydrolases (EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25), usually are involved in the modification or degradation of plant cell wall galactomannans [64,66].

These results imply that α-D-galactosidase could play an important role in seed germination, and activity of this enzyme may indeed influence germination rate and percentage. It has been unknown so far whether α-D-galactosidase activity is related to the changes in germinability and vigour of long-stored seeds containing RFOs and Gal-C. To investigate such possible relationships, the seeds of *Vicia hirsuta* (tiny vetch, Fabaceae) have been chosen. These seeds contain RFOs and galactosyl derivatives of *myo*-inositol and D-pinitol, that belong to galactinol, GPA and GPB series [50,67,68]. Seeds of this species, due to their unique composition of soluble α-D-galactosides, can be considered as a suitable model for studies on metabolism and the role of RFOs and Gal-C in legume seeds. During seed maturation, the content RFOs and Gal-C increases, reaching 4.5–6.9% of seed mass in mature, dry seeds [50,69].

The aim of this study was to investigate a relationship between the activity of α-D-galactosidase and the germination and vigour parameters of seeds *V. hirsuta* subjected to storage at 22 °C for different time periods. The seeds were stored up to 25 years. In addition to α-D-galactosidase activity, the analysed parameters were: maximum percentage of germination (G_{max}), time need to reach 50% and 75% of germination (t_{50} and t_{75}), germination rate ($1/t_{50}$ and $1/t_{75}$), germination uniformity (U_{8020}), seedling (epicotyl, root) length and seedling dry mass, Seed Vigour Index 1 (SVI 1) and Seed Vigour Index 2 (SVI 2). Furthermore, the possibility that a decrease in α-D-galactosidase activity could be an indicator of seed germinability and vigour changes in long-time stored seeds containing soluble RFOs and Gal-C was examined.

2. Materials and Methods

2.1. Plant Material

Seeds of *Vicia hirsuta* (L.) S.F. Gray (tiny vetch) were collected from their natural habitat in Olsztyn, Poland (53°43′ N latitude, 20°28′ E longitude) on July 1997. Seeds used in this study were at full maturity stage and with low water content <0.1 g g^{-1} dry weight. Seeds were placed in paper bags and kept at temperature 22 °C ± 1.5 °C, and 35% ± 5% relative humidity (RH) and stored for over 25 years, till 2022. Randomly chosen seeds, immediately after harvest (storage time 0 years) and after 1, 3, 5, 7, 9, 10, 11, 15, 20, 22 and 25 years of storage, were used for seed vigour and α-D-galactosidase activity assays. For the analyses of α-D-galactosidase activity, the seeds were frozen in liquid nitrogen immediately after being taken from storage bags at different storage time points, and stored at −86 °C until use.

2.2. Germination Tests and Assay of Seed Vigour Parameters

For germination tests, four replicates of 100 scarified seeds were placed on two layers of germination papers moistened with double deionized water in 160 mm glass Petri dishes. The seeds were arranged in ten rows of ten seeds each and incubated at 18 °C. The experiment was carried out with four replications. Seed germination was scored at 6 h intervals for 10 days to obtain accurate data and enable precise curve-fitting. Seeds were scored as germinated if the radicle protruded through the seed coat and reached at least 1 mm length. Since seeds of *V. hirsuta* are very small, stereoscopic microscope was used for germination scoring. Data on cumulative germination were used to estimate the following germination parameters: G_{max}, time needed to reach 50 and 75% of germination (t_{50}, t_{75}), germination rate ($1/t_{50}$ and $1/t_{75}$) and germination uniformity (U_{8020}), i.e., the time interval between 80% and 20% of seeds to germinate. To estimate these parameters, cumulative germination curves were fitted for each lot of stored seed, using the data of the cumulative increase in the percentage of seeds germinated during the germination time course. The cumulative germination curves were fitted using GraphPad Prism (ver.6). A four-parameter logistic regression (4PL, Hill's function, and the least square fitting method) was used for estimation models of cumulative seed germination over time. Based on model parameters, values of t_{20}, t_{50}, t_{75} and t_{80} were estimated by interpolation, and used for calculation of germination rate and uniformity parameters. Data are presented as the means ± SE of these values.

For seedling growth assessment, four replicates of 10 seeds were placed on moistened filter papers. The rolled papers were kept at 18 °C. After 10 days of germination, from each replication five seedlings were randomly chosen, and the length of epicotyl (EL), root (RL) and of whole seedling (SL) were measured. Additionally, those seedlings were dried in the oven at 103 °C for 17 h and weighed for seedling dry weight determination (SDW). Seed vigour indexes (SVI 1, SVI 2) were calculated per the following formulas, Seed Vigour Index 1 (1) and Seed Vigour Index 2 (2):

$$Seed\ Vigour\ Index\ 1 = germination\ (\%) \times average\ seedling\ length\ (cm) \quad (1)$$

and

$$Seed\ Vigour\ Index\ 2 = germination\ (\%) \times average\ seedling\ dryweight\ (mg). \quad (2)$$

Seed vigour indexes were calculated according to [24]. Data are the means ± SE of these values.

2.3. α-D-Galactosidase Activity Assays

A total of 100 seeds in four replications were used for soluble proteins extraction. Each sample was ground to a fine powder with a ball mill (Retsch, MM200, Haan, Germany) and approximately 100 mg of powder was extracted with 1.5 mL extraction buffer, composed of McIlvaine buffer (0.04 M citric acid, 0.1 M disodium hydrogen phosphate, pH 5.4), 1% (*m*/*v*) PVPP (Sigma-Aldrich, Poznań, Poland) and protease cOmplete inhibitor cocktail (Roche, Warsaw, Poland). Extracts were desalted and buffer was exchanged with HiTrap Desalting Columns 5 mL (Cytiva Global Life Sciences Solutions, Warsaw, Poland). α-D-galactosidse activity was assayed in 100 µL of reaction mixture containing 50 µL of McIlvaine buffer (pH 4.8), 0.5 mM *ρ*-nitrophenyl α-D-galactopyranoside (Sigma-Aldrich, Poznań, Poland) as substrate and 25 µL of the desalted, suitably diluted protein extract in McIlvaine buffer (pH 4.8). Reaction mixtures were incubated at 37 °C for 12 min and stopped by adding 180 µL of 0.5 M Ca_2CO_3. The amount of *ρ*-nitrophenol formed in the enzymatic reaction was quantified by measuring absorbance at 410 nm with a microplate reader (Tecan, Infinite® 200 PRO, Tecan Group Ltd., Männedorf, Switzerland). The control mixture involved adding enzyme extracts after the stop solution had been added. These controls were used as the zero calibration reading. Protein content was quantified by Bradford method [70], using Coomassie protein assay reagent (Sigma-Aldrich, Poznań, Poland) and

using bovine serum albumin (Sigma-Aldrich, Poznań, Poland) as a standard. The activity of α-D-galactosidse was expressed in nmol min^{-1} mg^{-1} (nmol of ρ-nitrophenol formed during 1 min of incubation by mg of seed protein at 37 °C). Data are the means ± SE of these values.

2.4. Statistical Analysis

Statistical analysis were performed using Statistica 13.0 software (StatSoft, Kraków, Poland) and GraphPadPrism 6 software (for Windows, GraphPad Software, San Diego, California USA). Data were analysed using Repeated Measures ANOVA (one-way analysis of variance, Tukey post-hoc test). Differences between means of seed vigour parameters or enzyme activities were considered to be significantly different at the $p < 0.05$ level. To assess the relationship between α-D-galactosidse activity and vigour parameters of stored seeds, Pearson correlation coefficients were estimated. Data on germination percentage, t_{50}, t_{75} and U_{8020} were arcsine-transformed to meet assumptions of parametric tests (normality and homogeneity of variances) before ANOVA and correlation analyses were done.

3. Results and Discussion

3.1. Germination and Vigour Parameters of Long-Stored Seeds

To investigate the effect of long-term storage on changes in germination ability and seed vigour parameters, the seeds of *V. hirsuta* with high initial germination were used. Germination at 99.25% (Figure 1A) indicates high viability of used seeds. Like many other species of Fabaceae family, *V. hirsuta* produces seeds with physical dormancy imposed by seed coats that are impermeable to water [71]; thus, seeds were chemically scarified with 98% H_2SO_4 for 45 min. before used in all seed vigour tests.

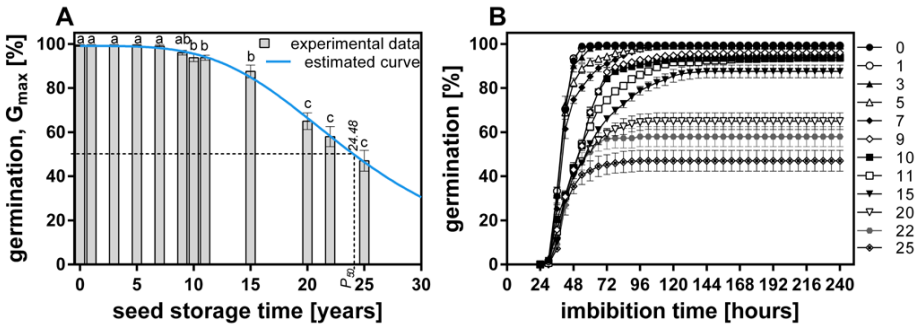

Figure 1. Effects of long-term storage of *V. hirsuta* seeds on: (**A**)—final germination percentage G_{max} and (**B**)—cumulative germination. Seeds were stored under ambient storage conditions (22 °C and 35% RH) for up to 25 years. P_{50} value was estimated using sigmoidal function. Each data point represents the mean ± SE of four replicates. Different lowercase letters indicate significant differences under different storage time ($p < 0.05$, Repeated Measured ANOVA, Tukey test).

Germination tests showed a progressive decrease of germination percentage (G_{max}) depending on the storage duration. Initial high germinability remained unchanged for a long time and a statistically significant decrease of G_{max} was found only after 10 years of storage. Germination percentage declined sharply between 15′ and 20′ years of storage, when G_{max} dropped to 65%. After 25 years of storage, G_{max} was 47%, indicating that at least 47% of seeds were still viable.

The results show that *V. hirsuta* seeds remained viable and able to germinate after a long storage period. Decrease of their germinability by a half occurred only after 25 years of storage. Ewart [8] found that *V. hirsuta* seeds were not able to germinate after 45 years of storage. Seeds of *V. hirsuta*, like many others of Fabaceae species, belong to the orthodox storage category; they become dehydrated during maturity and in this state they can survive for long periods of dry storage, resuming growth on rehydration. The viability of stored orthodox seeds can be prolonged by storing at cool and dry conditions, while

seeds tend to deteriorate faster at high temperatures, high relative humidity and high seed moisture content [3,72]. A systematic review on long term storage and longevity of orthodox seeds pointed out that many species of Fabaceae family produce long-lived seeds [11]. However, the duration of their longevity varies between species and strongly depends on storage conditions [3,73]. Justice and Bass [74] analysed P_{50} values that reflect the time of storage at which 50% or more of seeds can be expected to germinate. They found that seeds of a few crop species of *Vicia* genus, can be stored under ambient conditions for longer than 5 years (*V. villosa*, *V. angustifolia*, *V. dasycarpa*). Other studies reveal that P_{50} values for seeds of cultivated species of *Vicia* genus stored under ambient conditions were 10.8–11.4 years [9,13]. Under more optimal storage conditions (cool/cold, dry or genebank conditions), the values of P_{50} for cultivated *Vicia* spp. were several times higher and ranged between 84.99 and 144.25 years [15,75], indicating that *Vicia* spp. belong to the plants forming long-lived seeds [11]. Results of this study suggest that seeds of *V. hirsuta* are characterized by a life span longer than other crop species of *Vicia* genus, when stored under ambient conditions.

Despite the fact that *V. hirsuta* seeds maintained a high ability to germinate after long periods of time (Figure 1), their vigour did decline during storage (Figure 2). It was indicated by an extended time needed to reach 50% and 75% of germination (t_{50}, t_{75}) and extension of the period needed for the germination to rise from 20% to 80% (U_{8020}). Values of t_{50}, t_{75} and U_{8020} remained unchanged during the first 7 years of storage, thereafter their values increased significantly. After 15 years of storage, values of t_{50}, t_{75} and U_{8020} increased approximately 47, 98, and ~600%, respectively, compared to initial seeds (0 years of storage time), implying that the germination proceeded slower and slower.

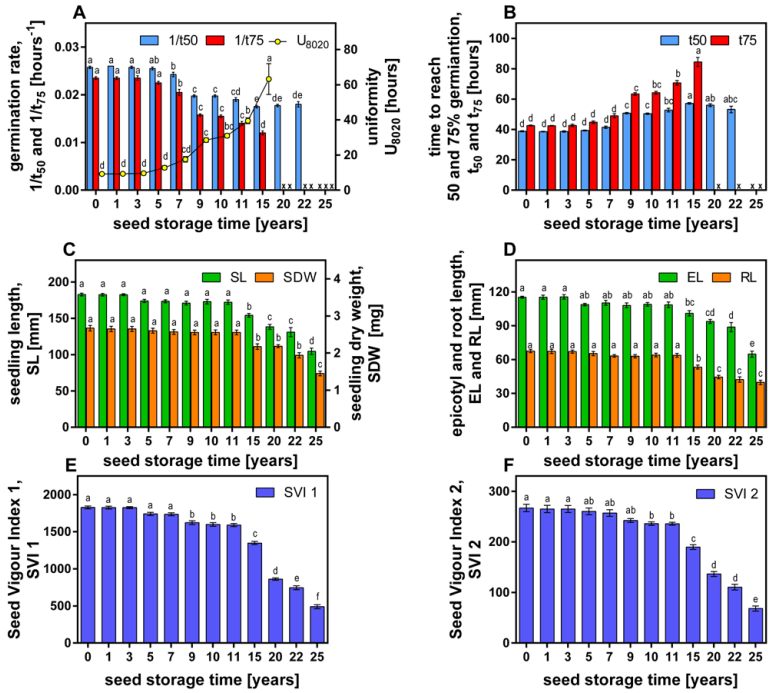

Figure 2. Effect of long-term storage of *V. hirsuta* seeds on: (**A**)—germination rate parameters $1/t_{50}$ and $1/t/_{75}$ [hours $^{-1}$]; uniformity of germination, U_{8020}: time interval between 20% and 80% of seeds to germinate [hours]; (**B**)—time needed to 50% and 75% seeds to germinate: t_{50} and t_{75} [hours]; (**C**)—seedling length, SL [mm] and seedling dry weight, SDW [mg]; (**D**)—epicotyl (EL) and root length (RL) [mg]; (**E**)—Seed Vigour Index 1, (SVI 1); (**F**)—Seed Vigour Index 2, (SVI 2). Each data point represents the mean ±SE of four replicates. For each seed trait, different lowercase letters indicate significant differences in the trait between storage times ($p < 0.05$, Repeated Measured ANOVA, Tukey test). Each of x means single data lack, "×" due to too low seed germination percentage estimation of values was not possible.

Reduced germination rate [h^{-1}] led to an extended period of time needed to complete germination of stored seeds, which was manifested by parameters $1/t_{50}$ and $1/t_{75}$ (Figure 2A). Values of $1/t_{50}$ and $1/t_{75}$ remained unchanged for 5 years and decreased significantly just after 7 years of storage. As storage time increased, the germination rate progressively declined.

These results indicate that the decrease of *V. hirsuta* seed quality during long-term storage resulted in a reduction in germination rate and increase in time needed to reach a given percentage of germination (Figure 2B). Furthermore, a progressive decrease in seed vigour was revealed by the reduced values in epicotyl (EL), root (RL) and seedling lengths (SL), seedling dry weight (SDW) and seed vigour indexes (SVI 1, SVI 2). The values of these parameters declined in a storage time-dependent manner (Figure 2).

Seed vigour indexes for the first years of storage decreased only slightly; however, with longer storage time, they decreased gradually, and their values reduced by half between 15 and 20 years of storage (Figure 2F). After 25 years of storage, SVI 1 and SVI 2 decreased considerably, up to ~25% of their initial values.

SL, SDW, EL and EL remained unchanged up to 11 years of storage, i.e., longer than did the seed vigour indexes. Afterwards, these values decreased significantly; although, even in seeds stored for 25 years, these parameters remained at a level that was over 50% of their initial values (Figure 2C,D). This indicates that tiny vetch seeds can maintain, not only high germination capacity, but also high vigour for a long time. In seeds of *V. hirsuta* stored for the longest time, seedlings length and weight reduced only by ~40% compared to seedlings raised from the initial seeds.

Changes in values of vigour parameters t_{50}, t_{75} and U_{8020} of long-stored *V. hirsuta* seeds followed an S-shaped curve, whereas values of $1/t_{50}$, $1/t_{75}$, SL, SDW, EL, RL, SVI 1 and SVI 2 exhibited an inverted S-shaped curve, which followed the same pattern as changes in G_{max}. Similar curves have been found to adequately describe the viability loss during seed storage in other species [76–78]. S-shaped curves comprise of three phases, the plateau phase (Phase I), followed by the rapid decreasing phase (Phase II), and the slow decreasing phase (Phase III) [77]. While changes in vigour parameters followed the same pattern in this study, the length of the first lag phase, when statistically significant differences were not found, differed among the individual parameters. Germination rate was the most sensitive quality trait of stored seeds, and a decrease of $1/t_{50}$ and $1/t_{75}$ values after 7 years of storage was the first sign of time-induced changes in seed vigour. In long-term stored seeds of *V. hirsuta*, changes of vigour parameters ($1/t_{50}$, $1/t_{75}$, SVI 1, SVI 2 and t_{50}, t_{75}, U_{8020}) preceded a significant decrease of seed viability, as measured by G_{max}. This sequence of changes is common to ageing seeds of many species [17]. When seeds deteriorate during ageing, they first lose vigour and the rate of their germination and seedling size declines, then the ability to germinate falls and, finally, the seeds die. Lowering of vigour and loss of viability results from chemical processes in seeds and from progressive accumulation of irreversible deteriorative changes. Loss of seed viability is the final stage in seed deterioration [79].

3.2. Activity of α-D-Galactosidase of Long-Stored Seeds

To investigate effect of storage on activity of α-D-galactosidase, mature seeds of *V. hirsuta* were long-time stored at ambient storage conditions (22 °C and 35% RH). In seeds aged for different time periods, the changes of enzyme activity were examined. For the first 3 years of storage, α-D-galactosidase activity remained unchanged; then, up to 11 years of storage, activity decreased negligibly (Figure 3A). A statistically significant decrease in activity was found in seeds stored for 15 years and longer. After 25 years of storage, α-D-galactosidase activity was reduced by ~30% (Figure 3).

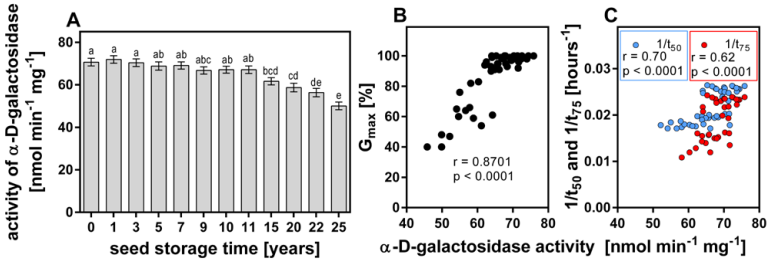

Figure 3. Effect of long-term storage of *V. hirsuta* seeds on activity of α-D-galactosidase (**A**). Relationship between α-D-galactosidase activity and final germination percentage (**B**) and germination rate (**C**). *V. hirsuta* seeds were stored at 22 °C and 35% RH for up to 25 years. Enzyme activity was assayed with synthetic substrate ϱ-nitrophenyl α-D-galactopyranoside and the amounts of released *ρ*-nitrophenyl was measured. Activity is expressed as nmol *ρ*-nitrophenyl formed during 1 min by 1 mg of seed protein extract at 37 °C. Each data point represents the mean ± SE of four replicates. Different lowercase letters indicate significant differences in activity between storage times ($p < 0.05$, Repeated Measured ANOVA, Tukey test).

3.3. Correlation between α-D-Galactosidase Activity and Germination of Long-Stored Seeds

Statistically significant positive correlations between activity of α-D-galactosidase and parameters of *V. hirsuta* seed vigour whose values decreased during storage, G_{max}, germination rate $1/t_{50}$ and $1/t_{75}$, seedling vigour indexes, seedling length and dry weight can be seen in Table 1. The highest Pearson correlation coefficients ($p < 0.0001$) were between enzyme activity and G_{max}, SL, SVI 1 and SVI 2. Enzyme activity was negatively correlated with germination uniformity (U_{8020}) and time needed to reach 50 and 75% of germination (t_{50}, t_{75}) (Table 1). These results indicate that changes in seed α-D-galactosidase activity were associated with seed ageing and reduction in vigour.

Table 1. Correlations between seed vigour traits and α-D-galactosidase activity of *V. hirsuta* seeds stored at 22 °C and 35–40% RH up to 25 years.

Trait	G_{max}	$1/t_{50}$	$1/t_{75}$	t_{50}	t_{75}	U_{8020}	EL	RL	SL	SDW	SVI 1	SVI 2	AGAL
G_{max}	1	0.82 ****	0.88 ****	−0.80 ****	−0.87 ****	−0.86 ****	0.84 ****	0.88 ****	0.89 ****	0.83 ****	0.96 ****	0.95 ****	0.87 ****
$1/t_{50}$		1	0.99 ****	−0.98 ****	−0.97 ****	−0.89 ****	0.70 ****	0.70 ****	0.72 ****	0.67 ****	0.77 ****	0.76 ****	0.70 ****
$1/t_{75}$			1	−0.99 ****	−0.98 ****	−0.92 ****	0.67 ****	0.65 ****	0.75 ****	0.57 ****	0.92 ****	0.82 ****	0.62 ****
t_{50}				1	0.98 ****	0.91 ****	−0.68 ****	−0.68 ****	−0.70 ****	−0.65 ****	−0.75 ****	−0.75 ****	−0.67 ****
t_{75}					1	0.97 ****	−0.66 ****	−0.71 ****	−0.77 ****	−0.62 ****	−0.95 ****	−0.87 ****	−0.65 ****
U_{8020}						1	−0.63 ****	−0.74 ****	−0.77 ****	−0.63 ****	−0.94 ****	−0.87 ****	−0.68 ****
EL							1	0.87 ****	0.98 ****	0.95 ****	0.91 ****	0.91 ****	0.83 ****
RL								1	0.96 ****	0.90 ****	0.94 ****	0.93 ****	0.85 ****
SL									1	0.96 ****	0.95 ****	0.95 ****	0.87 ****
SDW										1	0.90 ****	0.94 ****	0.82 ****
SVI 1											1	0.99 ****	0.89 ****
SVI 2												1	0.87 ****
AGAL													1

Statistical significance is indicated by asterisks, **** p (two-tailed) < 0.0001. G_{max}, maximal germination [%]; $1/t_{50}$ and $1/t_{75}$ germination rate [hours^{-1}]; t_{50} and t_{75}, time to reach 50 or 75% germination [hours]; U_{8020}, uniformity of germination [hours]; EL, epicotyl length [mm]; RL, root length [mm], SL, whole seedling length [mm]; SDW, seedling dry weight [mg]; SVI 1, Seed Vigour Index 1; SVI 2, Seed Vigour Index 2; AGAL, activity of α-D-galactosidase of *V. hirsuta* seeds stored for different time at ambient conditions [nmol min^{-1} mg^{-1}].

These findings are consistent with a previous study on biochemical changes during ageing of safflower seeds. Those seeds were subjected to controlled deterioration conditions (50 °C and 60% RH) and changes in metabolites and enzyme activity were detected. It was found that seed α-D-galactosidase activity decreased with seed ageing. Statistically significant declines in enzyme activity were found in seeds that germinated at 75%. These changes were accompanied by an abrupt raffinose content reduction [78]. However, opposite results were obtained in a study on α-D-galactosidase activity changes during storage of common beech seeds. It was found that decreases in germination capacity of beech seeds during long-term storage at −10 °C, were associated with an increase in α-D-galactosidase activity [31]. This discrepancy in pattern of α-D-galactosidase activity changes in stored seeds of *V. hirsuta*, safflower and common beech may be associated with fact that common beech seeds belong to the intermediate category of seeds [80], unlike the orthodox safflower

and *V. hirsuta* seeds. Accordingly, the storage conditions used for common beech seeds were different from those applied to the orthodox seeds. Summarizing, results of this study and a former study conducted on safflower seeds clearly indicate that during storage of orthodox seed, α-D-galactosidase activity decreases.

Reduction, or complete loss of enzymatic activity is an often observed symptom of seed ageing. When seeds deteriorate, many essential enzymes are inactivated, and among them are enzymes capable of degrading the stored reserves. During seed ageing, enzymes like many other proteins, undergo a variety modifications (folding, unfolding, fragmentation, aggregation), and their composition may change due to loss or gain of various functional groups and small compounds. Enzymes may also undergo a non-enzymatic glycosylation [3,40,81–84]. These modifications may lead to protein damage and degradation resulting in loss of functional properties of seed proteins and enzymes. This, in turn, can result in reduced protection against ROS and decreases in ATP production and respiratory substrates or intermediates [3,78,85]. Reduction in metabolic energy may result both from lowered activity of respiratory enzymes as well as from low activity of enzymes hydrolysing storage oligosaccharides. The final outcome is slower seed germination [78]. Moreover, enzyme inactivation in aged or long-stored seeds, together with damages to the genetic material and organelles, may lead to cellular dysfunction and finally loss of viability [3].

High activity of α-D-galactosidase supports fast seed germination. This is because this enzyme hydrolyses the soluble α-D-galactosides and cleaves terminal galactose moieties from RFO and Gal-C molecules, resulting in energy for germination (Figure 4). Respiration during initial phase of germination requires a supply of readily available substrates other than those derived from hydrolysis of the major stored reserves. In legumes, these substrates are galactose molecules released by α-D-galactosidase hydrolysing RFO and Gal-C molecules. However, it is worth noting that the high content of monosaccharides, including galactose, in stored seeds does not promote seed longevity. This phenomenon probably results from reducing properties of monosaccharides and their involvement in protein destruction in Maillard's reaction [31,44,81]. It has been shown that galactose levels have a highly significant negative correlation with the germination percentage of seeds under various aging treatments [86]. For successful germination, during the imbibition phase of germination, the amount of galactose released is crucial, but the total quantity of free galactose in stored seeds is not.

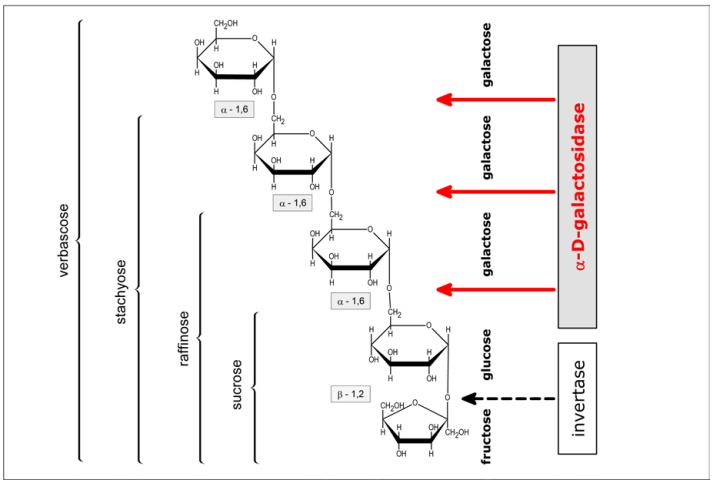

Figure 4. Scheme of α-D-galactosidase action on RFO molecules in germinating seeds. The trisaccharide raffinose is the simplest sugar of the raffinose family oligosaccharides, containing one α(1–6)-linked galactosyl moiety; stachyose contains two galactosyl moieties and verbascose has three. Hydrolysis of α-1,6-*O*-glycosyl bond results in release of terminal galactose residues from RFOs. Based on [56,87], modified.

Studies conducted on pea seeds (storing RFOs) and winter vetch (storing RFOs and Gal-C) proved that exogenously applied specific α-D-galactosidase inhibitor (1-deoxygalactonojirimycin, DGJ) decreased the seed germination rate. In germinating pea seeds, DGJ blocked RFO breakdown and reduced germination rates to approximately 25% of control, two days after imbibition [57]. Likewise, in winter vetch seeds, DGJ inhibited RFO degradation and delayed seed germination by 50% during the first three days of imbibition [88]. Similarly, when maize seeds were imbibed in the presence of DGJ they exhibited decreased seed germination percentages, but surprisingly increased seed ageing tolerance [4]. Constitutively overexpressing *ZmAGA1*, which encodes alkaline α-D-galactosidase 1, decreased both RFO and galactinol contents of mature, desiccated seeds of Arabidopsis (desiccated or undergoing imbibition), yet enhanced the seed germination percentage under either salt or osmotic-stress conditions [4]. This result implies that α-D-galactosidase plays an important role in seed germination and its activity influences germination rate.

A reduction in the amount of galactose released from RFO during germination, either as a result of low galactosidase activity or low galactosides content in the seed, results in a reduction in the rate of germination, but does not completely inhibit germination. Reduced amounts of available energy during germination did not significantly decrease the germination percentage of aged seeds [78]. It may be that metabolic energy is be released by conversion of other energy sources. This may also explain why germination percentage was not significantly reduced in pea seeds with a reduced level of RFOs as a result of α-D-galactosidase overexpression [89]. However, it is unknown how this reduction of RFOs content in seeds influenced their rate of germination. A high germination rate is crucial for field establishment, especially for crop seeds.

In recent years, many seed ageing markers and many new methods have been proposed to identify changes associated with seed ageing (reviewed by Fu et al. [90]). Modern methods can be an alternative to germination-based methods [86,91,92], but they are often expensive and require specialised equipment. The results of this work indicate that a decrease in α-D-galactosidase activity may be a good indicator of deterioration of stored seeds. Determinations of the activity of this enzyme measured using synthetic 4-nitrophenyl α-D-galactopyranoside are fast, accurate and give reproducible results. They are also inexpensive and do not require specialised testing equipment. They can, therefore, be used to assess quality changes in stored seeds.

In conclusion, results of this work indicate that seeds of *V. hirsuta* are characterized by a long life span, even when stored under ambient conditions. During long-time storage, activity of α-D-galactosidase decreases. This change is correlated with loss of seed germinability and reduction in seed germination rate and seedling growth parameters. Decrease of α-D-galactosidase activity in long-stored seeds may be a marker of reduced seed viability and vigour. Estimation of changes in galactosidase activity should be considered as quick and cheap method for evaluation of changes in stored seed quality.

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