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STORAGE AND AGEING OF FRENCH BEANS (*Phaseolus vulgaris* L.): EFFECT ON SEED VIABILITY AND VIGOR*

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ABSTRACT

The loss of viability and vigor compromises the agricultural usefulness of seeds and can also affect their nutritional properties. In this work, we examined the viability and vigor of freshly harvested French beans and of beans stored at approximately 8°C for 12 years. Quiescent seeds and seedlings grown until cotyledon abscission were subjected to germination and vigor tests, as well as microscopic analysis. As a control, fresh seeds underwent accelerated ageing for 8 and 16 days, and showed a gradual loss of viability. The stored seeds contained less humidity (6.5 ± 0.3%) than fresh seeds (12.9 ± 1.2%), with an imbibition time that increased from 6 h to 25 h. Germination began 24 h later and reached 86.8 ± 5.4%, which was not significantly different from that of fresh beans (96.0 ± 3.5%). The frequency of shooting and the rate of daily growth were only slightly better for fresh beans grown in the dark. Morphological analysis revealed alterations in the crystalline structure of starch in stored seeds that differed from those seen in beans with accelerated ageing. The mobilization of reserves occurred in fresh and stored beans. In addition, in stored beans and after accelerated ageing, there were alterations in the arrangement of proteins, and many cell walls were ruptured. The acid phosphatase activity was similar in fresh and stored beans, but decreased after 8 days of accelerated ageing and was not detected after 16 days. These finding suggest that enzymatic activity can be used as a bio-marker for seed viability and vigor.

Key words: Cytochemistry, French beans, germination, seed ageing, storage

INTRODUCTION

Adequate seed storage is a significant agricultural problem because of the need to maintain seed viability and vigor [24], particularly in tropical regions with a high humidity. In general, seeds can be classified as recalcitrant (lose their viability within a few days) or orthodox (remain viable for a long time). As an example, the storage of maize seeds (considered to be orthodox seeds) at a temperature and relative humidity higher than 25°C and 65-80% respectively, for more than 4 months, may be harmful [1].

Accelerated ageing (AA) is an important procedure for understanding the events that lead to the loss of seed viability. AA damages DNA and mRNA [23], causes biochemical deterioration of the stored material [18], and reduces the vigor of seedling and early plantlet development shortly after germination [4]. The consequences of ageing on cooking properties, digestibility, and formation of resistant starch in seeds have also been investigated [14]. However, little is known about the cellular alterations, storage mobilization and vigor during early plantlet growth [21].

At a cellular level, aged *Phaseolus vulgaris* seeds show a significant increase in the number and a decrease in the size of starch grains, as well as rupture of the cell walls and membrane-bound organelles, including protein bodies. Accelerated ageing of these seeds resulted in a loss of seed viability after 16 days or 3 years of storage at 25°C and 65% of relative humidity [5].

The objective of this study was to examine the cellular alterations during ageing and initial plantlet development in *P. vulgaris* seeds, including the changes in acid phosphatase activity, an enzyme involved in signal transduction and that has been used as a bio-marker in animal cells [3,10].

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*This paper is dedicated to Professor Benedicto C. Vidal on the occasion of his 75th birthday.

MATERIAL AND METHODS

Plant material and ageing

Seeds of *P. vulgaris* L. cv. Carioca that had been freshly harvested or had been stored at approximately 8°C in a refrigerator for 12 years were used. After surface sterilization (2% HClO, 5 min), part of the fresh material was artificially aged by exposing five groups of 20 seeds to 42°C and 100% relative humidity [9] for 8 or 16 days. The fresh and dry weights of 50 seeds in each category were measured to determine the seed humidity [5].

Imbibition curves

Ten samples of five seeds from fresh and stored material were weighed and imbibed in distilled water. After 1, 2, 3, 4, 5, 6, 7, 12, 18, 24, 30 and 36 h, the samples were weighed again and returned to the same flask. The imbibition curve was expressed as the percent weight increase relative to the imbibition time (in hours).

Germination test

Five samples of 50 seeds from each group (fresh, stored and artificially aged seeds) were germinated at 25 ± 1°C in gerboxes lined with paper towels soaked in distilled water containing 100 units of Mycostatin® solution/ml and maintained in the dark. Radicle emergence was considered indicative of germination and was evaluated daily for 14 days. The mean germination time (MGT) was calculated according to Santana and Ranal [19].

Growth analysis and vigor

Fresh and stored seeds, and seeds that had undergone accelerated ageing (AA) for 8 days were used for growth analyses. The seeds were kept in the dark or on a 12 h photoperiod. Six pots with vermiculite containing ten seeds for each different material were used to estimate the initial growth development. The materials were sprayed with Mycostatin® (100units.ml^{-1} in distilled water). Shoot lengths were measured until cotyledon abscission and the growth was expressed as the change in size relative to the time after germination. Twice a week, the plant with the median length of each population was sampled for microscopic analyses.

In situ analysis

Cotyledons were fixed in a 5% formaldehyde and 5% glacial acetic acid in 70% ethanol solution, for 48 h at 5°C [13]. The material was dehydrated in ethanol (70% - 100%), cleared in xylene for 10 min, embedded in paraffin and sectioned (7 μm thick). After removing the paraffin, part of the material was stained with 0.025% toluidine blue in McIlvaine buffer, pH 4.0, for 15 min [22], and the remainder was stained with 0.1% xylidine ponceau in 3% acetic acid for 15 min, according to Cortelazzo and Vidal [8]. A Polzeiss microscope was used to examine the slides, with or without a cross analyzer and polarizer.

Carbohydrate quantification

Sugars were extracted with a mixture of methanol, chloroform and water (12:5:3, v/v/v). After homogenisation, the samples were centrifuged twice (10 min, 3,000 x *g*) and the supernatant was used as the sugar-rich soluble fraction. The pellet was resuspended in 10 ml of 30% perchloric acid and centrifuged three times as described above [17]. The soluble material represented the starch-rich fraction. The sugar content of the various supernatants was quantified by the anthrone method using glucose (Sigma) as the standard [20]. The above procedure was performed three times.

Preparation of a crude extract for acid phosphatase activity

Forty seeds from each group were homogenized in 100 ml of 100 mM sodium acetate buffer, pH 5.0, and the homogenate was centrifuged at 10,000 x *g* for 20 min at 4°C and filtered through four layers of cheesecloth [11]. The protein content was determined by the Bradford dye-binding technique [7] with BSA (Sigma) as the standard.

Acid phosphatase assay

The enzyme activity towards pNPP (p-nitrophenyl-phosphate), β-gly (β-glycerophosphate) and TyrP (tyrosine-phosphate) was assayed by measuring the amount of phosphate released in a reaction mixture (1 ml) containing 100 mM sodium acetate buffer (pH 5.0), 5 mM substrate and enzyme after a 20 min incubation at 37°C [11]. For the quantification of inorganic phosphate, the reactions were stopped by adding 1 ml of 3% (w/v) ammonium molybdate (in 200 mM acetate buffer, pH 4.0) followed by 0.1 ml of 120 mM ascorbic acid (in the same buffer). The color was developed for 30 min and the absorbance was read at 700 nm. The amount of inorganic phosphate produced was calculated using a molar coefficient of 4000 M^{-1} cm^{-1} [16].

Statistical analysis

The results were expressed as the mean ± SD, where appropriate. Statistical comparisons were done using the Tukey test for multiple comparisons at $P < 0.05$.

RESULTS

Comparison of the fresh and dry weights of the different seeds used showed a net decrease in the humidity of stored seeds. However, the dry mass remained the same after prolonged storage. When seeds were submitted to AA, the dry mass decreased and the fresh mass increased relative to the fresh material, although the humidity after AA was approximately 60% (Table 1).

Imbibition tests showed that the curve for freshly harvested seeds had an exponential profile, with maximum imbibition occurring after almost 6 h. In

contrast, stored seeds had a sigmoidal imbibition curve and the maximum imbibition occurred only after 18-24 h (Fig. 1A).

The germination of fresh seeds began after 24 h of imbibition and continued to 72 h to reach 96.0 ± 3.5% (mean germination time = 46 h). For stored seeds, initial germination was delayed, with significant germination

Table 1. Fresh and dry weights of French beans before imbibition.

Seeds	Fresh weight (mg)	Dry weight (mg)	Humidity (%)
Freshly harvested	263 ± 24 c	229 ± 22 a	12.9 ± 1.2 b
12 years of storage	232 ± 13 d	217 ± 12 a	6.5 ± 0.3 c
8 days of AA	476 ± 29 a	193 ± 9 b	59.4 ± 2.4 a
16 days of AA	385 ± 30 b	150 ± 16 c	61.0 ± 3.1 a

The values are the mean ± SD of ten samples of five seeds. **AA**, accelerated ageing. Means followed by the same letter in each column do not differ significantly (Tukey test).

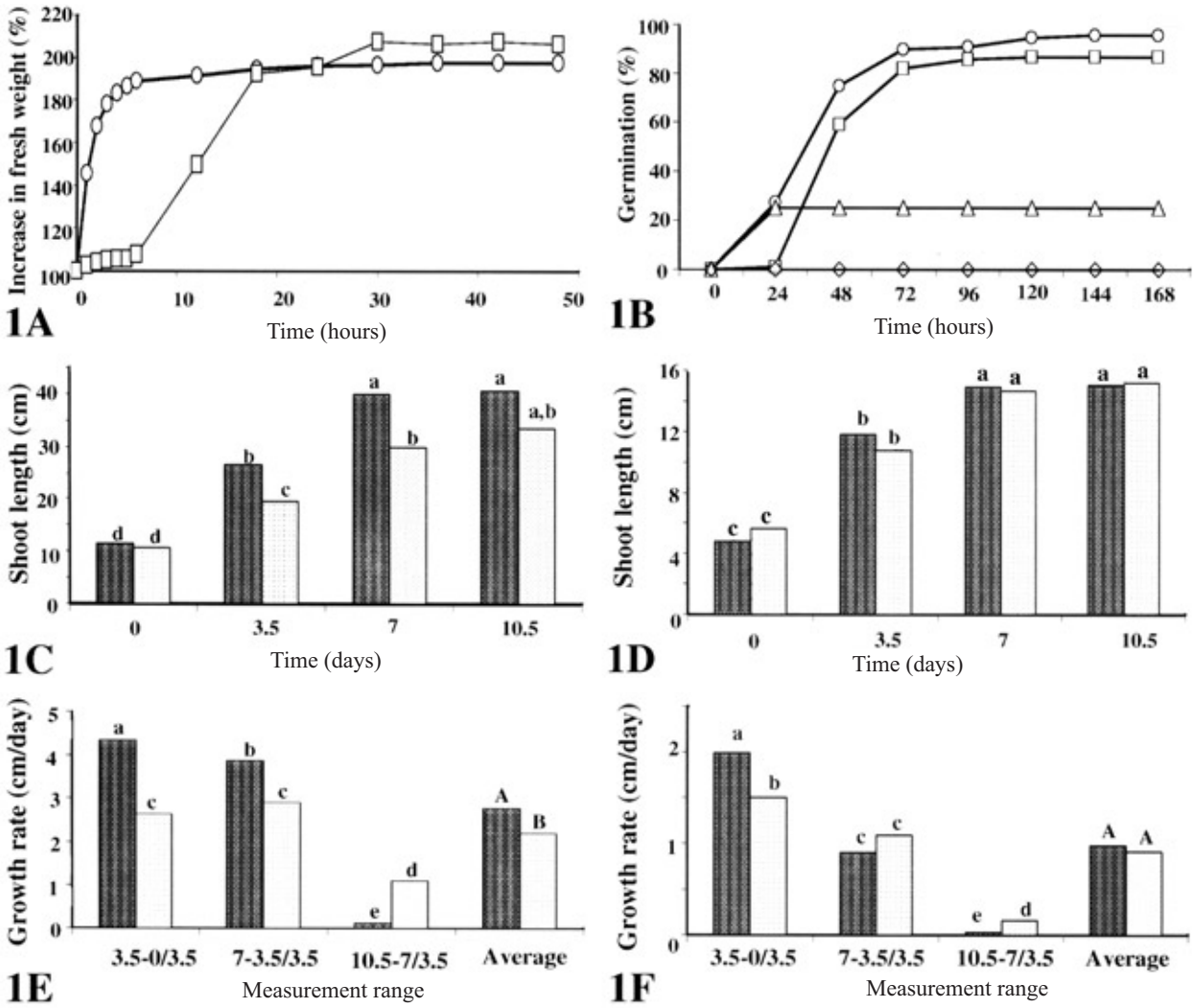

Figure 1. Changes in various parameters of *P. vulgaris* seeds during ageing. **A:** Increase in fresh weight during water imbibition, (-0-) freshly harvested seeds, (-□-). **B:** Germination, (-o-) freshly harvested seeds, (-□-) 12 years old stored seeds, (-Δ-) 8 days of accelerated ageing (AA), (-◊-) 16 days of AA. **C-D:** Shoot length (cm) of plantlets grown in the dark (**C**) or 12 h photoperiod (**D**). **E-F:** Growth ($cm.day^{-1}$) in the dark (**E**) or on a 12 h photoperiod (**F**). (■) freshly harvested seeds, (▭) seeds stored for 12 years.

occurring only after 48 h of imbibition (mean germination time = 57 h), to reach 86.8 ± 5.4% (Fig. 1B).

Seedlings from fresh seeds grown in the dark had longer shoots than seedlings from stored seeds but, after cotyledon abscission, the shoot lengths in both groups were not significantly different (Fig. 1C). The shoots of seedlings kept on a 12 h photoperiod were shorter than for those grown in the dark, but were similar for fresh and stored seeds (Fig. 1D). Plantlets of fresh seeds kept in the dark grew more during the first week and remained larger than stored seeds (Fig. 1E). In contrast, seedlings from fresh and stored seeds kept on a 12 h photoperiod showed the same growth after one week (Fig. 1F).

In sections of cotyledons from fresh seeds, staining with xylidine ponceau revealed proteinaceous material surrounded by slightly elliptical, unstained starch grains. Xylidine ponceau positive material was also seen inside phloem elements (Fig. 2A). The same results were obtained with toluidine blue, which stained anionic elements, in addition to the cell walls (Fig. 2B). Polarized light microscopy revealed birefringent cell walls and the typical "Malta cross" structure in starch grains, which were present at a high concentration in the seeds (Fig. 2C,D), as confirmed by quantitative analysis (Table 2).

The cotyledon cells of stored seeds showed a less organized structure in their protein reserves (Fig. 2E,F) and the starch grains, with the "Malta cross" being less evident (Fig. 2G,H). The degree of disorganization varied for the protein reserves, but not for the carbohydrate reserves (Table 2).

Seeds subject to AA showed a loss of protein content and were more disorganized than stored seeds. After eight days of AA (Fig. 2I-K), cellular degradation began, with loss of cell content and a clear decrease in the diameter of the starch grains (Fig. 2J,K), when compared to freshly harvested (Fig. 2C,D) and stored seeds (Fig. 2G,H). This aspect was accentuated after AA for 16 days, the time necessary for total loss of viability and cell death. The cell rupture and protein loss were more evident at this interval (Fig. 2L), as was the decrease in the volume of most of the starch grains (Fig. 2M,N).

Sections of cotyledon cells from an early plantlet (7 days) showed that the reserves were slightly depleted. This reduction was also seen in toluidine blue stained sections, although not as clearly (Fig. 3A). The typical "Malta cross" structure of starch grains was also seen with polarized light (Fig. 3B,C). Cotyledon abscission started two weeks after germination, but only after the 18th day did the last plantlets lose their cotyledons. In material collected 18 days after germination, microscopic analysis revealed a total depletion of the cotyledon reserves (Fig. 3D,E).

In stored seeds, the mobilization of part of the reserves after one week of development revealed the alterations in the crystallinity of starch grains more clearly (Fig. 3F,G). However, these plantlets lost their cotyledons in the same period as plantlets from fresh seeds, with total reserve depletion in the peripheral (Fig. 3H) and medial (Fig. 3I) regions, including the starch reserves (Fig. 3J).

The development of plantlets kept on a 12 h photoperiod was similar to, but faster than that of plantlets kept in the dark. In plantlets from fresh (Fig. 3K,L) and stored (Fig. 3M,N) seeds, cotyledon abscission without any reserve began 10 days after germination. No cotyledons were observed after 14 days.

Figure 4 shows the phosphatase activities of the different seeds. The enzymatic activity of stored seeds was generally slightly higher than for freshly harvested and 8-day-AA seeds. The enzyme activity towards pNPP was greater than for the other substrates. No enzyme activity was found after 16 days of AA (Fig. 4).

DISCUSSION

The results of this study showed that, despite the very long storage period, the seeds of *P. vulgaris* were nearly as viable as the freshly harvested seeds.

Table 2. Carbohydrate and protein content of freshly harvested French beans and after 12 years of storage.

Seeds	Free sugars (mg/g)	Starch (mg/g)	Protein (mg/g)
Freshly harvested	30 ± 0.7 a	457 ± 19 a	175 ± 5 a
12 years of storage	29 ± 0.7 a	469 ± 26 a	147 ± 5 b

The values are the mean ± SD of three different samples. Means followed by the same letter in each column do not differ significantly (Tukey test).

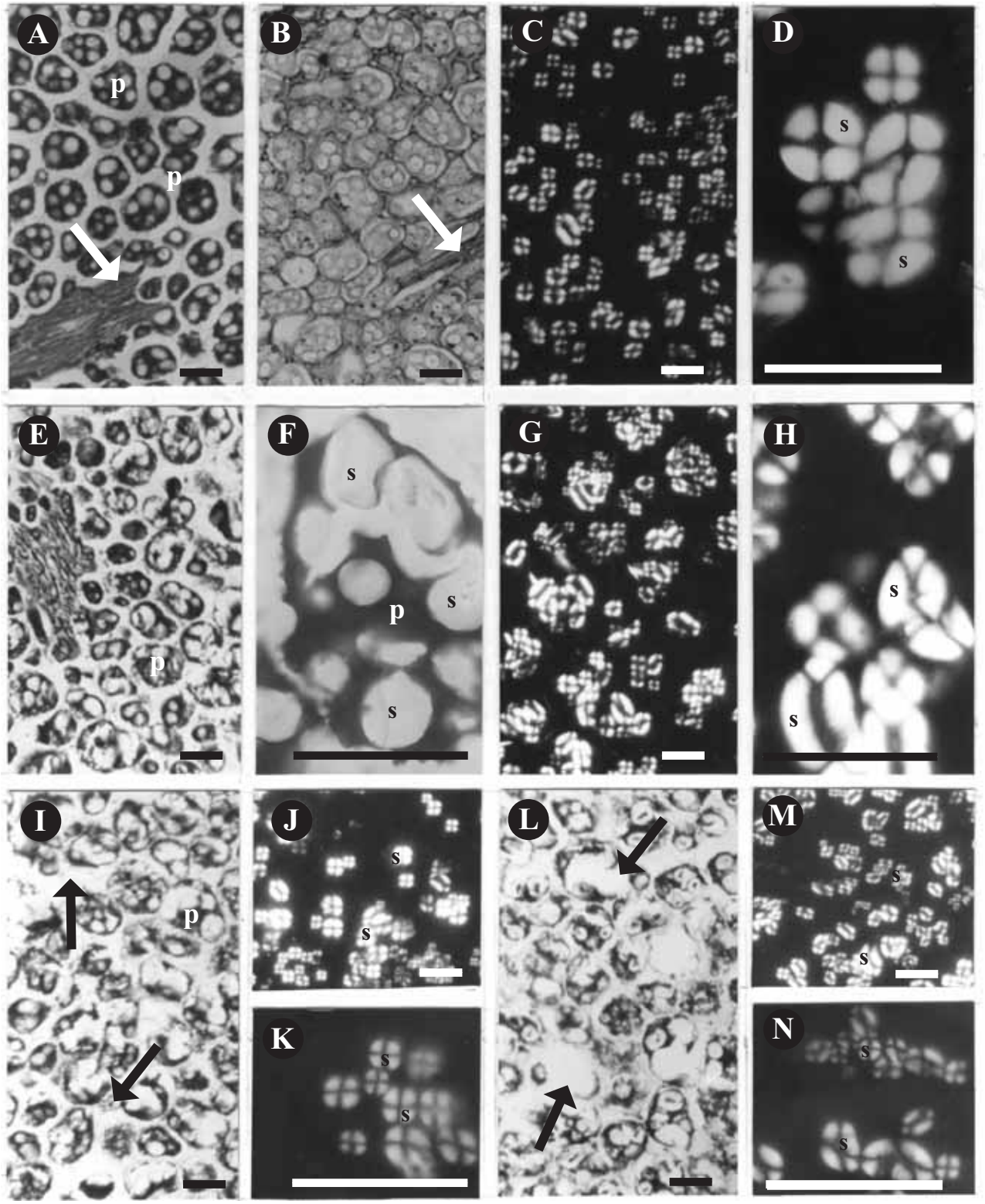

Figure 2. Sections of *P. vulgaris cotyledons*. **A-D:** Freshly harvested seeds. **E-H:** Seeds stored for 12 years in a refrigerator. **I-N:** Seeds after 8 days (**I-K**) and 16 days (**L-N**) of accelerated ageing **(AA)**. **A, E, F, I** and **L:** Xylidine ponceau-stained sections. **B:** Toluidine blue-stained sections. **C, D, G, H, J, K, M** and **N:** Polarized light microscopy. Note the stained material inside the phloem elements **(white arrows)**, the protein bodies **(p)**, broken cell wall **(black arrows)**, and characteristic "Malta cross" image of starch grains **(s)**. Note also the deformation of the starch grains after storage, with an increase in small grains, particularly after AA. Bars = 75 μm.

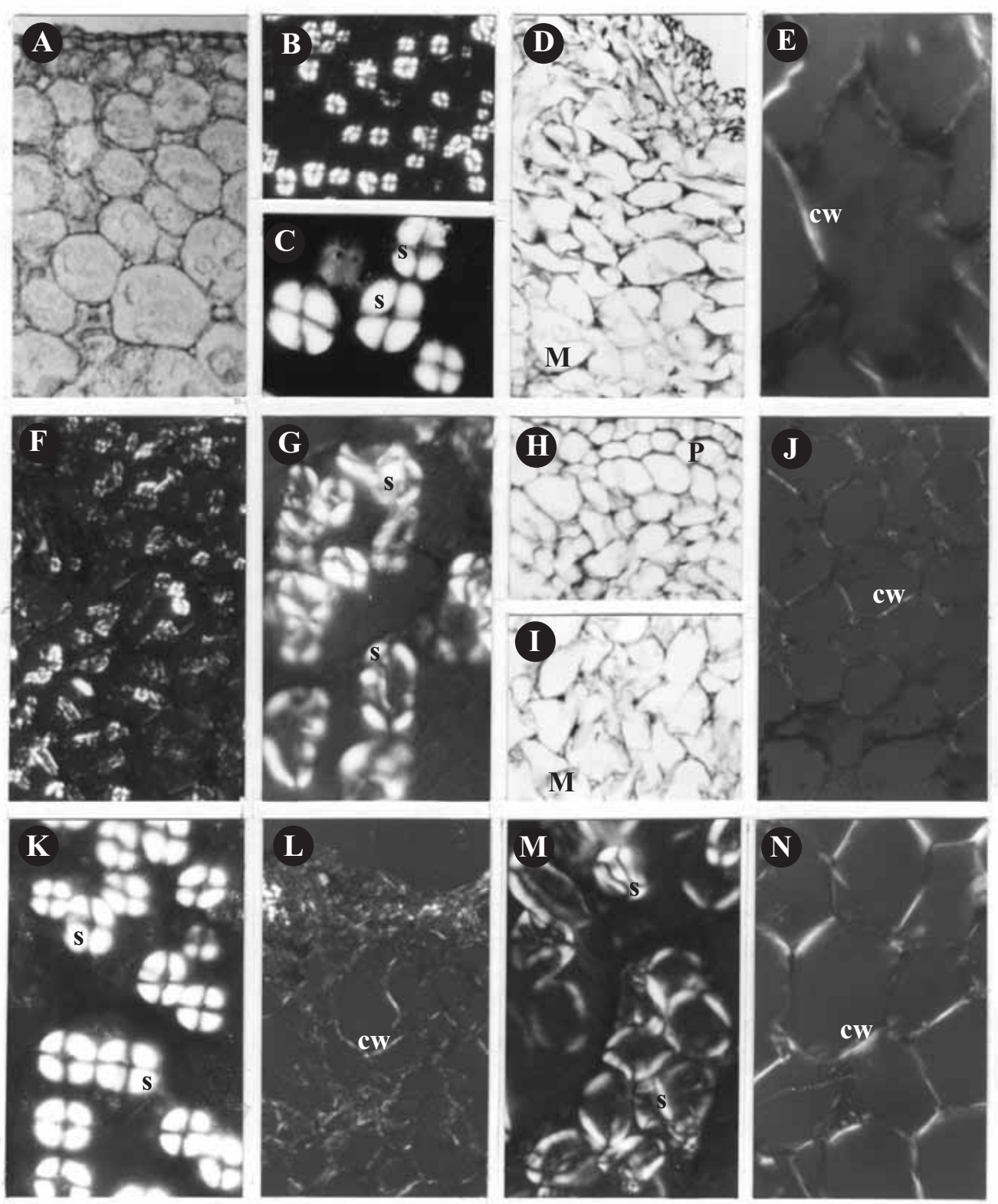

Figure 3. Sections of *P. vulgaris* cotyledons after germination and initial plantlet growth. **A-J:** Development in the dark. **K-N:** Development on a 12 h photoperiod. **A-E** and **K-L:** Freshly harvested seeds. **F-J** and **M-N:** Seeds stored for 12 years in a refrigerator. **A-C, F, G, K** and **L:** 7 days after germination. **D, E, H-J, M** and **N:** immediately before cotyledon abscission. **A, D, H** and **I:** Toluidine blue stained sections. **B, C, E-G** and **J-N:** Polarized light microscopy showing the cell wall **(cw)** and starch **(s)** birefringence. Note the deformation of the starch grains after storage and the depletion of total food reserves before cotyledon abscission in both the peripheral **(P)** and medial **(M)** cotyledon regions. Bars = 75 μm.

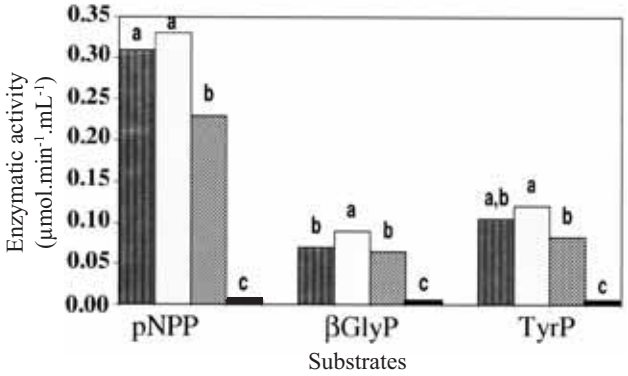

Figure 4. Acid phosphatase activity of freshly harvested seeds ( ), seeds stored for 12 years ( ) and seeds after 8 days ( ) and 16 days ( ) of accelerated ageing (AA). **pNPP -** p-nitrophenylphosphate, **βGlyP** - β-glycerophosphate, **TyrP -** tyrosine-phosphate. Columns with the same letter for each substrate do not differ significantly (Tukey test).

The seeds lost only about half of their humidity during this period (from 13% to 6.5%) since they had been stored in closed containers. An increase in hardness and a decrease in water permeability were observed, leading to an increase in the imbibition time. A similar result was obtained during natural ageing or storage at high temperatures, which produced greater tegument hardness and, in many cases, a delay in imbibition [14].

The marked decrease in seed viability after 8 days of AA and their inviability after 16 days confirmed previous results obtained for these beans [5]. However, even for plantlets germinated from AA seeds, the final shoot length was the same (data not shown). For seeds kept in the dark, there was a large increase in seedling shoot length that was slightly greater for fresh seeds. For seeds kept on a 12 h photoperiod, the aerial parts were shorter than for those grown in the dark, although seed age had no influence on this response. This difference was attributed to a greater growth rate in plantlets kept in the dark, especially for fresh seeds. In contrast, seedlings kept on a 12 h photoperiod were more vigorous and healthier than those kept in the dark.

Acid phosphatase can be extracted from seed cotyledons and is detectable before germination in several species such as castor bean [12], soybean [2], lentil [6] and squash [15]. The similar profile of enzymatic activity in fresh, stored and accelerated aged seeds suggested that the chemical and three-dimensional structures of this enzyme were maintained. The lack of enzyme activity after 16 days of AA coincided with the death of the seeds, and suggested that this enzyme can be used as a biomarker of seed viability.

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