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Biochemical Changes in *Pinus pinea* Seeds During Storing

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The changes in the germination-rate, the contents in germination-inhibitors and the biochemical differences in soluble proteins and nucleic acids in freshly harvested *Pinus pinea* seeds stored for various periods of time, up to 24 months, and at two different temperatures (room temperature and 4° C), have been investigated. The present results show that the maturation or after-ripening process of this type of seeds might be induced during the first 6 and 12 months of storage. However, seeds stored for longer periods of time might also be thought to enter into the primary phases of the ageing process where early alterations occur, including the loss of germination-rate and germination-inhibitor contents in the seed coat, together with an incapacity for the seeds to increase their protein and nucleic acid levels during the germination process.

It has been known that seeds of different plant species vary greatly in their life span and that usually their viability decreases with the time of storage after ripening where the seed moisture content and seed temperature play an important role (11).

The ageing of seeds is related to the decrease of their ability in germination and viability. This effect becomes a problem in modern agricultural practice (17). The mechanism of ageing or deterioration is still an enigma, but it is imperative to understand this process in order to provide conditions to slow down the ageing, or prescribe safe seed storage conditions.

Most of the reported studies have been carried out on cereal, leguminous and other seeds (1-3, 9, 10, 19, 20, 22) but very little is known about the process taking place in seeds with high lipid content as in the case of *Pinus pinea* seeds.

In this paper the biochemical differences in pine seeds stored for several

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periods of time under two different kinds of temperature conditions are reported.

Materials and Methods

Pine seeds (*Pinus pinea* L.) from Coca (Segovia, Spain) were used throughout this work. They were kindly supplied by the Forestry Institute of Madrid.

Storage conditions. Two lots of recently collected *P. pinea* seeds were stored at 4° C and room temperature (18-25° C) respectively. Samples were taken at different periods of the storage time (0, 6, 12 and 24 months). Two other lots assayed were only stored at room temperature during 27 and 35 years.

Germination test. Pine seeds were surface sterilized in a 1 % (w/v) solution of sodium hypochlorite for 10 min, rinsed thoroughly in sterile distilled water and dried between layers of absorbent paper. One hundred sterilized seeds were partially buried in sterile vermiculite at 26° C under continuous fluorescent light, using at least four replicates, each of them with 100 seeds. Vermiculite and seeds were moistened with 1 ml distilled water per seed at the beginning and halfway through the experiments.

Protrusion of radicle through the seed coat was used as criterion for germination. The data are expressed in percentage of germination after 50 days of sowing. Similar germination conditions were applied to obtain samples of germinated seeds for further assays. The seed coat and membranes of each seed were removed and the endosperm and embryo were isolated manually.

Extraction of seed germination-inhibitors and their bioassay. Inhibitors of seed germination from pine seed coats were extracted and bioassayed as described (15).

Extraction of simple proteins and their determination. Albumins, globulins and prolamins were extracted by the method of GRZESIUK and KULKA (10) and the concentration determined by the method of LOWRY *et al.* (12). Albumins were fractionated on Sephadex G-100 column, 45 × 2.5 cm ∅, and Sephadex G-200 column, 90 × 2.5 cm ∅. Protein solutions (10-15 mg) in 2-4 ml were layered onto the column. The samples were eluted with phosphate buffer (0.01 M, pH 7), at a flow rate of 10-15 ml/h. Fractions (2 ml) were collected by using a LKB fraction collector. The absorption at 280 nm of the various fractions was carried out using an UNICAM SP 1800 spectrophotometer.

Extraction of nucleic acids and their determination. RNA and DNA were extracted by the method of OGUR and ROSEN (18). The concentrations of the nucleic acids were determined spectrophotometrically at 260 and 280 nm and expressed as mg/100 g of dry weight (8).

RNase and DNase activities were determined by measuring spectrophotometrically the absorption of the breakdown products which were not precipitated by 0.3 % uranylacetate in 0.2 M of perchloric acid (13).

Results and Discussion

Germination. Pinus pinea seeds were stored at room temperature and at 4° C for 2 years. The percentage of germination after 50 days of sowing increased gradually during the first 12 months of storage, especially in seeds stored at room temperature, and the germination percentage decreased as the period of storage increased (table I). However, it should be noted that these changes were the results of alterations in the rate of seed germination, and did not correlate with the germination capacity since increasing the time of seed sowing up to 80 days the final percentage of germination was in all cases

Table I. *Effect of time and temperature during storage on* P. pinea *seed germination (expressed in % after 50 days of sowing).*
Percentage of germination in vermiculite at 26° C in continuous light. Mean figures are the results of four determinations.

Storage period	% Germination	
	Room temperature	4° C
0 months	10 ± 1	10 ± 1
6 months	24 ± 2	19 ± 1
12 months	79 ± 5	49 ± 5
24 months	53 ± 4	32 ± 4
27 and 35 years	0	—

85 % and 70 % in seeds stored at room temperature and 4° C respectively. These data suggest that freshly harvested and stored *P. pinea* seeds may enter into the ripening process after 6-12 months storage, but longer periods of time (24 months) cause a decrease in the germination rate, probably when entering into the ageing process. This effect is more notably shown when seeds were stored at room temperature. However, longer periods of time are necessary in order to get a complete loss of viability, since 0 % germination was only observed in seeds stored at room temperature for 27 and 35 years whose germination percentage represents 95 % of the seeds original germination.

Germination-inhibitors. There is evidence that water-soluble germination-inhibitors are involved in the regulation of seed germination and dormancy. These inhibitors appear to be located in the coat, embryos and other seed tissues (5-7, 15, 21, 23). In order to study the possible variation of the inhibitors present in pine seed coats during periods of storage and their role in the viability of the seeds, these substances were extracted from our biological material and indirectly determined by bioassay on lettuce seeds. The effects of the seed coat extracts on lettuce seed germination are listed in table II.

Table II. *Effect of seed coat extracts on lettuce seed germination (expressed in % after 5 days sowing).*
Percentage of germination was tested at 26° C under light with 100 seeds sown on Whatman No. 1 filter paper in a Petri dish (9 cm ∅), containing 1 ml seed coat extract + 1 ml of distilled water or 2 ml distilled water (Control). Mean figures are the results of six determinations.

	% Germination		
	6 months	12 months	24 months
Control	96±4	99±1	98±2
Room Temperature	30±2	86±4	79±5
4° C	24±1	40±5	60±3

It may be observed that the inhibitory action, from inhibitor substances, present during the first six months of storage, at 4° C and at room temperature, decreased after 12 months, especially when stored at room temperature, persisting after this period of time at room temperature, but after two years at 4° C there was a slight decrease. It is possible that storage at room temperature stimulates the disappearance of germination-inhibitors, and the data would thus suggest that storage would improve the germination process especially during the first twelve months, as confirmed by the germination test. However, this is not the case when seeds were stored for a longer period of time where inhibitory action from seed coat's extracts appeared to be lower, although there was also a decrease in the germination rate of those seeds. It should be taken into account that inhibitors are not the only substances regulating the germination process.

Soluble proteins. Changes in the protein contents in *P. pinea* seeds were investigated at various ages, 0, 6, 12, 24 months of storage at 4° C and room temperature, paying particular attention to

Table III. *Effect of storage at room temperature on protein fractions of* P. pinea *endosperm (E) and embryo (e) in dry and germinated seeds (expressed in g % dry wt.).*
Mean figures are the results of three determinations.

Period of storage (months)	Conditions	Albumins		Globulins		Prolamins		Sum of proteins	
		E	e	E	e	E	e	E	e
0	Dry	6.8±0.17	9.6±0.32	7.0±0.74	3.7±0.25	0.5±0.21	0.3±0.00	14.3±1.12	13.7±0.57
	Germinated	22.2±3.65	17.5±1.42	2.8±0.33	3.6±0.10	0.3±0.04	0.8±0.10	25.2±4.02	21.9±1.62
6	Dry	7.3±0.37	12.5±0.43	8.4±0.58	11.7±0.63	0.1±0.00	0.2±0.00	15.8±0.95	24.4±1.06
	Germinated	18.8±0.01	10.5±0.08	5.3±0.00	10.7±0.16	0.2±0.00	0.2±0.00	24.2±0.01	21.5±0.24
12	Dry	4.1±0.46	8.2±0.54	6.9±1.57	4.5±0.31	0.2±0.03	0.3±0.07	11.1±2.06	13.0±0.92
	Germinated	17.3±4.25	8.8±1.88	4.2±0.29	1.8±0.55	0.9±0.19	1.8±0.52	22.3±4.73	12.3±2.95
24	Dry	4.9±0.43	9.4±1.06	5.7±0.52	2.9±0.24	0.5±0.00	1.3±0.20	11.1±0.95	13.6±1.50
	Germinated	7.6±0.70	4.6±0.90	5.2±1.00	4.5±0.01	0.7±0.11	1.5±0.34	13.5±1.81	10.6±1.25

Table IV. *Effect of storage at 4° C on protein fractions of* P. pinea *endosperm (E) and embryo (e) in dry and germinated seeds (expressed in g % dry wt.).*
Mean figures are the results of three determinations.

Period of storage (months)	Conditions	Albumins		Globulins		Prolamins		Sum of proteins	
		E	e	E	e	E	e	E	e
0	Dry	6.8±0.17	9.6±0.32	7.0±0.74	3.7±0.25	0.5±0.2	0.3±0.00	14.3±1.12	13.7±0.57
	Germinated	22.2±3.65	17.5±1.42	2.8±0.33	3.6±0.10	0.3±0.04	0.8±0.10	25.0±4.02	21.7±1.62
6	Dry	6.0±0.49	7.6±0.54	8.4±0.52	6.7±0.12	0.2±0.00	0.6±0.00	14.5±1.01	14.9±0.66
	Germinated	11.1±0.01	6.8±0.72	2.7±0.01	2.0±0.00	0.3±0.00	0.6±0.01	14.0±0.02	9.3±0.73
12	Dry	4.6±0.55	8.7±0.08	6.7±0.34	3.6±0.35	0.3±0.00	0.6±0.04	11.6±0.89	12.9±0.47
	Germinated	6.9±0.37	4.1±0.73	4.2±0.27	2.2±0.54	1.1±0.33	2.2±0.37	12.2±0.97	8.5±1.64
24	Dry	8.3±2.13	10.6±0.14	5.5±0.93	3.8±0.50	0.8±0.07	1.1±0.01	14.7±3.13	15.5±0.51
	Germinated	7.4±1.39	4.4±0.22	4.7±0.00	0.7±0.01	0.8±0.01	2.1±0.00	12.9±1.40	7.2±0.23

soluble proteins, albumins, globulins and prolamins (tables III and IV). The present results indicate no significant changes in total soluble protein contents in either embryos or endosperms of dry seeds by increasing the storage time at 4° C or room temperature up to 24 months; except in embryos from seeds stored for 6 months at room temperature where the amount of total soluble proteins was increased, especially in globulin fraction. Seeds stored for 6 months at 4° C also show higher levels of globulins in embryos than their controls, fresh harvested seeds; however the value of total soluble proteins was not significantly increased. During germination of fresh harvested (0 months storage) seeds the sum of soluble proteins in embryos and endosperms increased almost twofold, mainly due to higher levels in the albumin fraction. However, the sum of soluble proteins in the stored seeds did not increase after germination. In some cases a decrease was also observed, especially after 6 and 12 months of storage particularly at 4° C, where endosperms and embryos from germinated seeds contained less soluble protein than the respective dry ones. When comparing endosperms or embryos from germinated seeds, a decline was observed in the soluble proteins when the storage period increased, whether at either 4° C or room temperature. This effect was mainly due to lower levels in the albumin fraction, accompanied in some cases by a small increase in the contents of prolamins and globulins. These results would therefore be in agreement with those obtained by GRZESIUK and KULKA (10) in oat seeds from different crops. These authors stated that high molecular weight albumin fractions decrease with the time of storage, whereas those with low molecular weight increase. Afterwards the albumin fractions from our biological material were then fractionated on a Sephadex G-100 column and Sephadex G-200 column in order to observe possible qualitative changes, but the profiles obtained were similar, indicating that at those periods of storage no qualitative changes were detectable by these techniques.

Nucleic acids. The changes of RNA and DNA contents (table V) in dry and germinating pine seeds over different periods and storage temperatures have been investigated. The results show a small decrease in RNA levels when increasing the periods of storage in either dry embryos or endosperms, and the DNA contents increase during the first 12 months of storage, followed by a decrease in DNA levels when storaged further. Several authors (4, 9, 14) have described increased contents of RNA and DNA in germinating viable seeds, and GRZESIUK and KULKA (9) decreased levels when seeds presented diminished viability. Similar results to those for viable seeds were obtained in our biological material. However, after 24 months of storage, the increase in nucleic acid contents throughout the germination process became slightly diminished in endosperms, and more clearly decreased in embryos. Notwithstanding, it is well known that RNase and DNase activities increase in viable endosperms during the germination process (16) but not in non-viable endosperms (9). However, we were only able to detect an increase in the hydrolytic enzymes, nucleases during germination in endosperms after 6 months of storage, and not after longer periods of time.

From the results obtained with the storage at 4° C or at room temperature for *P. pinea* seeds recently collected throughout the 12 months storage period, there exists an increase in the germination percentage; a decrease in the inhibitor content in the seed coats, and an increase in the levels of soluble proteins and nucleic acids in comparison to germinating seeds and dry seeds. For these reasons, it is thus possible to assume that they are at a primary stage of ripening.

Table V. *Effect of storage at room temperature and at 4° C on nucleic acid contents of* P. pinea *endosperm (E) and embryo (e) in dry and germinated seeds (expressed in mg % dry weight).*
Mean figures are the results of three determinations.

Storage Months	Conditions	RNA		DNA	
		E	e	E	e
Control	Dry	106.7± 4.38	371.8± 31.95	71.7± 1.26	252.8± 45.32
	Germinated	251.2± 0.01	630.2± 86.02	506.0±99.63	1897.7± 27.10
Room temperature					
6	Dry	70.2±14.87	274.9± 5.71	218.6± 2.51	609.6± 49.54
	Germinated	159.3±19.58	822.0± 16.32	576.2±84.03	2801.0±339.54
12	Dry	63.3± 0.00	217.5± 7.50	342.0±68.58	763.1±152.62
	Germinated	180.6±25.71	1013.4±196.69	540.0±99.34	3099.6±620.00
24	Dry	57.1± 0.00	178.8± 14.44	125.8±12.50	478.1± 79.70
	Germinated	156.9± 4.52	440.0± 26.11	255.3±15.69	1840.0±113.18
4° C					
6	Dry	57.5± 2.90	277.2± 20.32	241.9±26.42	583.7± 50.33
	Germinated	166.6± 0.01	404.0± 0.02	682.8± 0.10	2400.0± 2.40
12	Dry	85.4±37.75	214.2± 54.35	356.3±50.90	713.8± 92.76
	Germinated	186.5±50.65	990.6±130.59	396.1±47.76	3506.8±185.20
24	Dry	45.3± 1.81	159.0± 0.01	139.1±18.70	631.6± 62.88
	Germinated	93.3± 2.27	370.1± 38.30	390.6±15.78	1799.3± 22.13

Nevertheless, from the data obtained for longer storage periods (i.e. 24 months), the seeds might be thought to enter into the first phase of the ageing process where it is possible to note a decrease in the rate of germination, and an incapacity by the seeds to increase their protein and nucleic acid contents during the germination process. It would be probably necessary to have a much longer storage time in order to obtain more notable results, since in seeds storaged for 27 and 35 years there was not only a decrease in the germination rate but also in the percentage capacity of germination (i.e. in the seeds 27 and 35 years old the final percentages were reduced to zero).

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Resumen

Se estudian los cambios en la capacidad germinativa, presencia de inhibidores de la germinación, proteínas solubles y ácidos nucleicos en semillas de *Pinus pinea*, producidos por su almacenaje durante distintos períodos de tiempo, hasta un máximo de 24 meses, y a dos temperaturas (temperatura ambiente y 4° C). Durante los 12 primeros meses de almacenamiento parece inducirse el proceso de maduración, mientras que períodos más largos inician el envejecimiento, observándose pérdida de la germinabilidad y presencia de inhibidores de la germinación en las cubiertas de las semillas, así como descenso de la capacidad para incrementar sus niveles de proteínas solubles y ácidos nucleicos durante el proceso de la germinación.

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