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LOSS OF VIABILITY DURING STORAGE IS ASSOCIATED WITH CHANGES IN MEMBRANE PHOSPHOLIPID

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Abstract—Viability (in terms of *in vitro* germination), membrane integrity [as revealed by the fluorochromatic reaction (FCR) test] and changes in total and individual phospholipids were monitored in pollen grains of *Crotalaria retusa* stored under different conditions for up to 60 days. Irrespective of the storage conditions, there was a positive and significant correlation between the loss of viability and the loss of membrane integrity as well as the reduction in total phospholipid and individual phospholipids The results indicate that membrane phospholipid deterioration, and consequent loss of membrane integrity represent an important physiological event associated with the loss of pollen viability.

INTRODUCTION

Pollen grains lose viability over a period of time. This loss of viability is greatly affected by environmental conditions particularly the temperature and relative humidity. Although extensive studies have been carried out to prolong viability by storing pollen grains under different conditions, the causes for the loss of viability are not known. Based on circumstantial evidence, a deficiency of respiratory substrates and inactivation of enzymes have been suggested to be the causes for the loss of pollen viability [1, 2]. Recent studies by Heslop-Harrison and his associates [3, 4] have suggested that the loss of membrane integrity is primarily responsible for the loss of viability in pollen grains subjected to short-term desiccation. Our investigations on the storage of pollen grains in organic solvents have shown that membrane integrity is critical for maintenance of viability [5, 6]. In seeds of many species, loss of viability has been correlated with a decrease in total phospholipid as well as major phospholipid classes [7–9]. Phospholipid damage and the consequent membrane deterioration have been suggested to be the early events in the loss of seed viability [9]. There is no information on the changes in phospholipid in pollen grains during storage We have monitored viability, membrane integrity and changes in total and individual phospholipids in pollen grains of *Crotalaria retusa*, stored under different conditions, to find out the correlations, if any, between the loss of viability and the loss of membrane integrity and/or decrease in the levels of total and individual phospholipids.

RESULTS AND DISCUSSION

Fresh pollen samples showed 85–90% germination and produced *ca* 500 μm long tubes after three hr incubation at $27 \pm 2°$. Their fluorochromatic reaction (FCR) score was *ca* 90%. The results of viability tests on stored pollen are presented in Table 1. Under laboratory conditions (uncontrolled), there was a progressive and significant decline in pollen viability (as assessed by % germination) from day seven of storage with complete loss of viability by day 60. The tube length also showed significant reduction in stored pollen.

Under desiccated conditions, there was no reduction in % germination and % FCR during the first seven days of storage. Per cent germination showed a significant reduction from day 15 onwards and % FCR from day 30 onwards However, pollen tube length showed a significant decline from day seven. Thus, low humidity prolonged viability for 7–15 days. Even after 60 days of storage a proportion of the pollen showed viability.

Pollen stored at low temperature ($-20°$) for 30 days showed % germination and % FCR comparable to the control (fresh pollen). A significant decline in % germination and % FCR was observed only by day 60. The vigour of the pollen tube was not reduced even by day 60.

As reported for many other systems, high temperature and high humidity are detrimental for pollen storage [2, 10]. This is true for seeds also [11, 12]. An interesting finding in the present investigation is that even before the decline in FCR or germination scores, reduction in pollen tube growth rate became apparent especially under laboratory temperatures Until recently, the reduction in the vigour of pollen tube has not been taken into consideration in stored pollen [10, 13].

The correlation between germination and FCR scores was significant ($P < 0.01$). This reconfirms earlier studies on the importance of the integrity of pollen membranes for germination [3–6]

The data for total phospholipid and individual phospholipids in pollen samples stored for different periods under the three conditions are shown in Table 2. Storage at room temp. (uncontrolled condition) caused a progressive decline in total phospholipid content extracted from pollen throughout the storage period (Table 2). There was a significant decrease in total phospholipid when compared to the control even after seven days of

Table 1 Changes in germination, pollen tube length (μm) and FCR of *Crotalaria*

Storage conditions	7 days			15 days		
	% Germination ± s e	Pollen tube length (μm)	%FCR ± s e	% Germination ± s e	Pollen tube length (μm)	%FCR ± s e
Room temp (uncontrolled)	56 8* ± 5 36	412 72† ± 25 23	54 8* ± 2 44	38 4* ± 1 63	189 36* ± 5 93	20 5* ± 0 93
Room temp (desiccated)	84 8 ± 3 69	362 88* ± 22 73	89 4 ± 2 85	82 8* ± 1 65	382 28* ± 14 54	89 2 ± 3 52
−20	84 8 ± 2 69	492 76 ± 23 39	90 4 ± 3 17	92 8 ± 1 34	499 20 ± 31 76	90 8 ± 1 11

Fresh pollen samples (control) showed 89 34 ± 1 11% germination and produced 487 34 ± 20 37 μm long tubes after 3 hr incubation Their FCR score was 94 35 ± 1 05%

*Significant at $P < 0.01$ as compared to the control

†Significant at $P < 0.05$ as compared to the control

storage. In the sample stored for 60 days, which had lost viability completely, the reduction was over 40%

Pollen stored at room temperature under desiccation did not show any significant change in total phospholipid level up to 15 days There was a significant decline after 30 days of storage It appears that in pollen grains also, higher moisture content as well as extreme desiccation result in phospholipid damage similar to the reports on seeds [12, 14] Storage at −20° showed a significant decline in phospholipid level only after 60 days These results show a direct relationship between loss of viability during storage and the amount of extractable phospholipid present in *Crotalaria* pollen. The correlation between the two was statistically significant

The predominant phospholipids in *Crotalaria* pollen are phosphatidyl choline (PC) (56%) and phosphatidyl ethanolamine (PE) (26%) with lesser amounts of phosphatidyl glycerol (PG), phosphatidyl serine (PS) and phosphatidyl inositol (PI) Phosphatidic acid (PA) and lyso-derivatives were also detected but were not taken into consideration as they are considered to be artifacts due to hydrolytic activity during extraction [15]. There were significant changes in the composition of the phospholipid fractions in stored pollen. Phosphatidyl choline showed significant reduction in all the pollen samples which had lost viability partially or fully Maximum reduction was recorded in pollen samples stored under uncontrolled laboratory conditions, the reduction was *ca* 80% in the pollen sample stored for 60 days The reduction was comparatively less in samples stored at lower humidity under laboratory conditions and in those stored at −20°.

The reduction in PE was also similar to that of PC, although to a lesser extent There was no reduction in PE levels in pollen samples stored at −20°. Significant reduction in PG was observed only in samples stored under uncontrolled laboratory conditions for 30 and 60 days. There were no significant changes in the levels of PI and PS during storage under different temperatures and humidities

Phospholipids play a major role in the structure and function of the plasma membrane The present investigation clearly suggests that membrane phospholipid deterioration (as indicated by a decline in total phospholipid and in individual phospholipids, especially PC) and consequent loss of membrane integrity (as revealed by FCR test) represents an important physiological event associated with the loss of pollen viability Similar observations have been reported during ageing of seeds of many species [11, 16–20].

EXPERIMENTAL

Plant material Pollen grains were collected from freshly dehisced anthers of *Crotalaria retusa* L grown under field conditions The grains collected on each day were pooled and a small sample was used to determine % germination, pollen tube length and the membrane state The remainder of the sample was used to study storage under three conditions One sample was maintained under uncontrolled laboratory conditions (50–60% relative humidity and 18–25°), the second under laboratory temp but over dry silica in a desiccator (0–5% relative humidity) and the third sample was stored at −20° in a sealed container

Viability test Pollen from each storage condition was removed at suitable intervals and tested for viability using an *in vitro* germination test [5] in a medium containing boric acid (100 μg/ml), $Ca(NO_3)_2$ (300 μg/ml), $MgSO_4$ (200 μg/ml), KNO_3 (100 μg/ml) and sucrose (10%)

Pollen membrane integrity was assessed by the fluorochromatic reaction (FCR) test [21] Non-fluorescent fluorescein diacetate readily penetrates pollen membrane Esterases of pollen cytoplasm act on fluorescein diacetate and release fluorescein which traverses the intact membrane with great difficulty and so accumulates inside pollen cytoplasm where it can be detected by fluorescence microscopy The FCR test has been found to be satisfactory for the assessment of the integrity of the pollen membrane in many systems (see ref [4]) For each pollen sample 500 pollen grains (from five replicate cultures) were used to score % germination and % FCR, and 100 pollen tubes to measure tube length

Lipid extraction. Pollen grains were homogenized in 10 ml $CHCl_3$–MeOH (2 1) [22]. All solvents used contained 0 005% butylated hydroxytoluene to reduce auto-oxidation H_2O-soluble impurities were removed by partitioning overnight against 2 ml 0 9% NaCl soln The $CHCl_3$-MeOH phase was

pollen stored under different conditions

30 days			60 days		
% Germination ±s.e.	Pollen tube length (μm)	%FCR ±s.e	% Germination ±s.e.	Pollen tube length (μm)	%FCR ±s.e
13.6* ±1.18	203.56* ±10.71	6.2* ±0.93	0*	–*	0*
14.4* ±2.66	151.45* ±1.79	53.24* ±1.93	9.50* ±1.32	152.28* ±8.24	62.4* ±9.62
80.8 ±8.05	453.08 ±79.6	82.0 ±5.29	60.60* ±1.11	464.23 ±12.34	89.2† ±1.65

Table 2 Changes in the amount of total phospholipid and individual phospholipid classes extracted from pollen grains stored for different periods under different conditions

Storage condition/period	Total phospholipid	PG	PE	PC	PI ± PS
Fresh (control)	54.88 ± 1.35	4.75 ± 0.17	14.99 ± 0.20	32.15 ± 1.17	4.81 ± 0.24
Room temp 7 days	48.39 ± 0.72*	4.91 ± 0.08	11.78 ± 0.06*	24.57 ± 0.81*	5.25 ± 0.08
15 days	42.52 ± 0.64*	3.58 ± 0.58	12.88 ± 0.10*	21.18 ± 1.03*	5.25 ± 0.17
30 days	32.77 ± 1.02*	3.15 ± 0.46*	11.26 ± 0.56*	7.35 ± 0.27*	3.61 ± 0.57
60 days	30.31 ± 0.97*	2.28 ± 0.42*	10.03 ± 0.26*	5.70 ± 0.20*	4.49 ± 0.29
Room temp 7 days	54.55 ± 0.69	5.03 ± 0.19	15.35 ± 0.44	32.84 ± 0.47	5.06 ± 0.31
(desiccated) 15 days	50.21 ± 1.09†	4.65 ± 0.22	13.42 ± 0.64†	29.61 ± 0.27	4.12 ± 0.53
30 days	48.02 ± 0.60*	4.72 ± 0.13	12.24 ± 0.53*	23.21 ± 2.74*	4.32 ± 0.45
60 days	41.34 ± 0.95*	4.63 ± 0.18	12.31 ± 0.33*	21.21 ± 0.94*	4.26 ± 0.36
−20° 7 days	52.03 ± 0.95	4.41 ± 0.41	15.47 ± 0.29	33.03 ± 0.79	4.17 ± 0.52
15 days	52.10 ± 1.45	4.83 ± 0.32	14.57 ± 0.50	29.68 ± 0.87	4.25 ± 0.79
30 days	52.65 ± 0.84	4.68 ± 0.67	15.26 ± 0.12	29.23 ± 0.35	4.76 ± 0.58
60 days	48.16 ± 0.52*	4.74 ± 0.32	15.08 ± 0.37	25.57 ± 1.08*	4.78 ± 0.90

Quantity of phospholipids obtained by multiplying the value of phospholipid phosphorus by 25.
Values are expressed as mg/g dry wt and are means ± s.e. of three experiments with two replicates in each set.
*Significant at $P < 0.01$ as compared to the control
†Significant at $P < 0.05$ as compared to the control

evapd under N_2, the lipid residue dissolved in 2 ml of $CHCl_3$ and used to estimate total phospholipid [23].

Qualitative analysis Various phospholipid classes were separated by TLC (silica gel G, $CHCl_3$–MeOH–7 M NH_3 46 18 3) Phospholipids separated by TLC were visualized by means of I_2 vapour Identification of the different phospholipid spots were made by using specific spray reagents and by comparing R_f values with those of standards [24, 25]. Phospholipids were visualized with molybdenum blue reagent [26] Ninhydrin was used to identify amino phospholipids

Quantitative analysis. The areas of silica gel containing phospholipids were removed and following digestion with perchloric acid, the concn of each phospholipid was determined according to the method of ref [23].

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