EFFECTS OF POST-ANTHESIS STORAGE VARIABLES ON POLLEN OF COTTON (GOSSYPIUM HIRSUTUM L.)

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RESUMO

Problemas de tempo e espaço freqüentemente encontrados pelos geneticistas e melhoristas de algodão *(Gossipium hirsutum* L.) poderiam ser parcialmente resolvidos se o pólen pudesse ser armazenado sem perda de viabilidade. Vários métodos de armazenamento de pólen foram testados, visando estender sua longevidade. Procedimentos utilizados em estudos anteriores demonstraram que até 72 horas, em refrigerador doméstico, foi possível preservar a viabilidade do pólen de algodão. Para a determinação da viabilidade do pólen foram utilizados dois critérios, um *in vivo* (número de sementes por loja) e o outro *in vitro* (percentagem de germinação e comprimento do tubo polínico). No processo *in vitro* foi usada uma modificação do meio artificial idealizado por Taylor. No armazenamento, a temperatura de 5° C foi considerada melhor que a de 25° C. Quando submetido a qualquer processo de congelamento, o pólen antes do armazenamento preservou sua viabilidade em 5° C até um máximo de 72 horas. O nitrogênio, em substituição ao ar atmosférico, não foi capaz de estender a longevidade do pólen além das 72 horas previamente estabelecidas. Flor coletada antes da antese e armazenada por um período de 72 horas em 5°C, resultou em pólen viável. Como uma medida de viabilidade do pólen, os resultados in vivo e in vitro foram concordantes.

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PALAVRAS-CHAVE: Armazenamento de Polen de Algodão, Germinação de Polen, Semente por Lóculo, Crescimento do Tubo Polínico

SUMMARY

Time and space problems frequently encountered by geneticists and plant breeders who work with cotton, *Gossypium hirsutum* L., could be partially solved if cotton pollen could be stored without losing viability. A previously reported upper limit of 72 hours storage time was challenged by varying the degree of pre-storage desiccation and storage temperatura and atmosphere. Seed per locule was used as an *in vivo* criterion of pollen viability. Pollen germination percentages and tube length after a specified time interval were used as *in vitro* criteria of viability. Medium used was a modification of that used by Taylor. Storing pollen at 5°C was considerably better than at 25°C. Any form or freezing of freeze-drying of cotton pollen destroyed its viability. Desiccation prior to storage extended storage time at both 5°C and 25°C but not beyond the previous established 72 hour limit. Substituting nitrogen gas for normal air as storage atmosphere did not extend the time that cotton pollen could be stored. De-

hisced pollen lost viability rapidly, regardless of storage procedures. Flower removal prior to anthesis and stored up to 72 hours at 5°C did not reduce the viability of retained pollen. There was an excellent agreement between *in vivo* and *in vitro* measures of cotton pollen viability.

INTRODUCTION

Pollen of many crop plants can be stored for varying periods of time without losing viability. This permits crosses to be made between plants having different blooming on different continents. One exception is cotton (*Gossypium hirsutum* L.), which has a storage limit of approximately 72 hours. Efforts to extend the storage limit of cotton pollen requires practical criteria of pollen viability and vigor.

Pollen viability has been used synonymously with ability to germinate. Vigor of pollen has been used to represent rate of germination and subsequent tube growth. BARROW (4) has used viability to represent the pollen's potential ability to complete the fertilization process, a kind of summation term that includes either or both germination and vigor. Pollen viability is used in this sense here.

Pollen viability can be estimated *in vitro* or *in vivo.* The latter is limited by a four-to-six week flowering period in a normal growing season. HARRISON & FULTON (7), working with Pima cotton (*G. barbadense* L.), removed flower buds in the afternoon prior to anticipated anthesis and open flowers on the day anthesis and stored both buds and flowers at approximately 5°C for 48 hours with no reduction in the stored pollen's ability to complete the fertilization process *in vivo.* Pollen longevity was reduced by using loose pollen collected at 13: 00 hours on the day of anthesis. Storage temperature was limited by a domestic refrigerator. Loose pollen was weakened more with storage beyond 48 hours than pollen stored in the mature flower or flower buds and neither storage procedure was satisfactory beyond 72 hours.

In vivo methods require nine to twelve days after anthesis and potential fertilization before a first estimate of pollen viability can be made, unless tedious procedures suggested by BARROW (4) are followed. *In vitro* measures of cotton pollen viability, therefore, have potential interests among cotton breeders. Pollen sterility in cotton may be a response to environmental stress (4) and it may also be under genetic control (15). In either case pollen sterility is a general concern of cotton breeders. A continuing need exists for an effective method for estimating pollen viability in quantitative terms.

ASLAM et alii (2) used vital strains to estimate cotton pollen viability, with counts of 93-96% commonly encountered. This method could not be used to estimate pollen vigor. Other staining procedures (1, 3) have been used directly as a vigor measurement. BARROW (4) concluded that pollen effectiveness could be determined only by examining the lower part of the style for tube penetration of ovules.

Pollen that germinates can be considered viable but this does not assure adequate tube growth to complete the fertilization process (4). Pollen of cotton that has been stored may be weakened. The loss or retention of vigor becomes a companion concern with germination. Both germination and vigor criteria can be used to measure pollen storage effects.

Viability of pollen may be expressed *in vitro* as germination percentage and by other means. KEARNEY & HARRISON (9) obtained rapid and accurate estimates of pollen viability by inoculating droplets of a 5% sugar solution with fresh pollen. Those grains that exploded were considered viable. No comparative rates of tube growth could be made with this procedure. Cotton pollen germination procedures developed by BARROW (3) gave rapid and effective estimates of

pollen viability but could not be used to estimate vigor. His hanging drop procedure, however, resulted in germination estimates of 90 to 100%. MIRAVALLE (13) modified BRONCKER's (6) procedure for *in vitro* pollen germination and was able to obtain a generalized tube growth rate index; however, his techinique may not be sensitive enough to serve as a vigor discriminant when comparing different periods of pollen storage.

TAYLOR (15) developed an *in vitro* pollen germination medium that led to reliable estimates of viability. He also obtained estimates of vigor among cultivars, presumably all with good pollen. TAYLOR (loc. cit.) used agar supplemented with sucrose, manganous sulfate, calcium nitrate, and boric acid but was able to obtain germination percentages of only 30% as a general average. This can be compared to the 85 to 95% obtained by BARROW (3) and his hanging drop procedures. TAYLOR (15) obtained excellent measurements of tube growth from those grains that germinated. He aged agar plates at least 24 hours before seeding to avoid having the pollen grains sink into the surface of the agar or rupture because os excessive hydration. TAYLOR's procedures (loc. cit.) should be adequate to differentiate storage effects in cotton pollen.

Candidate procedures for storing cotton pollen include desiccation prior to storage, freezing or vacuum drying, and substituting nitrogen gas for normal atmosphere. RAO & GOVILA (14) reported the natural viability of cotton pollen to be 10 hours. HOEKSTRA & BRUINSMA (8) proposed that trinucleate pollen respires at a higher rate in humid air than does binucleate pollen. Cotton is presumed to have a trinucleate pollen grain according to botanical family listings of BREWBAKER (5). He suggested that trinucleate pollen having a higher respiration rate, may be sucrose or metabolite deficient at maturity. KIDD (10) used a substituted atmosphere for stored seed to reduce respiration. Prior desiccation may extend longevity in cotton pollen although HARRISON & FULTON (7) did not find this to be true.

Freeze-drying, sometimes referred to as lyophilization or vacuumdrying if dried under vacuum and frozen during the process, has extended pollen longevity in many crops according to KING (11). He has also suggested that holding freeze-dried pollen in an oxygen-free atmosphere gives an extended longevity by depressing respiration.

The ojective of this study was to seek ways of extending cotton pollen longevity beyond limits previously reported by utilizing procedures that have been used effectively for plants other than cotton.

MATERIALS AND METHODS

Arkugo 4, a cultivar of cotton *(Gossypium hirsutum* L.) that normally matures at Fayetteville Arkansas (36° 07' 30''N, 470 m elevation), was grown under field and greenhouse conditions to produce pollen used in this study. Storage duration variables were evaluated with single phase environmental variables of temperature, desiccation mode, freezing and thawing, and alteration of storage atmosphere.

Pollen for storage comparisons was from flowers collected at random after anthesis (after 0830 DST) except as noted specifically. The pollen from at least five flowers was used for each treatment. Initially, the dehisced pollen was stored over $CaCl_2$ at 25, 10, 5, and -10°C. After 0, 24, 48, 72, and 96 hours, subsamples were used for either an *in vivo* or an *in vitro* estimate of pollen viability.

In vivo estimates of pollen viability were made in terms of seed per locule. This was determined after the dehisced pollen was given its specific storage treatment and then was used to pollinate five randomly selected and tagged flowers that were among those emasculated the previous afternoon. Pollen treatment

effects with this procedure were not estimated until bolls matured approximately 60 days after fertilization.

Seed per locule was determined by removing mature bolls from plants grown in the field. Individual bolls were bagged and labelled and then stored until the seeds were counted. Seed counts were made of the first four locules encountered per boll. Many bolls had only 4 locules. Five bolls, tagged at random as described above, were usede to represent a treatment. Statistical analysis were based on a nested AOV.

In vitro estimates of pollen viability were made in terms of percentage germination on fortified agar and subsequent rate of pollen tube growth. TAYLOR's medium (15) was modified slightly after a series of single phase challenges for each constituent. We used 1.0% agar, 15.0% sucrose, 0.03% H_3BO_3, 0.06% $Ca(NO_3)_2$ and 0.09% $MnSO_4$ in distilled water and adjusted to pH 7.0. Pollen germination medium was prepared as suggested by TAYLOR (15). The mixture was heated to 100°C. Immediately after boiling, two drops of the solution were poured on concave slides. These were allowed to cool at room temperature and then stored for 24 hours at 5°C. Dehisced pollen from specified treatments was collected with a number 4 camel's hair brush and then gently brushed across the agar surface of the concave slide. A coverslip was placed on the slide, which was sealed with vaseline and placed on moistened filter paper in a petri-dish, which was then covered and held at 25°C. After 24 hours, germination and pollen tube growth were stopped by adding 1 or 2 drops of acetocarmine. Slides were stored at 5°C until counted. Subsequently, 25 pollen grains were examined in each quadrant of each of 4 slides per pollen treatment. The number germination if at least one recognizable tube was present. For pollen tube length, 25 randomly selected pollen tubes from each slide were measured and their lengths recorded. Germination counts and length measurements were made at a magnification of x 100.

Germination contrasts were evaluated with appropriate Chi-Square tests. Measurement data were evaluated with a nested AOV.

Temperature variables were maintained with standard mini-lab germination control chambers. Desiccation variables were maintained with 0.15 to 0.30g dehisced pollen being placed in small tubes loosely stoppered with sterilized tissure paper and held in standard laboratory desiccators with appropriate amounts of desiccative material in the bottom of the jar. In subsequent freezing and atmosphere substitution comparisons, a prior desiccation of 8 hours over $CaCl_2$ at 25°C was standard. Quick freezing was accomplished with a 5ml glass ampoule of pollen being immersed in a dry ice-acetone bath (−78°C) for 30 seconds. Each ampoule was frozen and held 24, 48, 96, and 120 hours before thawing by immersing in a 49°C water bath for 30 seconds. A portion of the quick frozen ampoules was attached to a mechanically referigerated freeze-dryer unit and the moisture removed under a vacuum of 250mm (mercury) for 15, 30, and 60 minutes. After freeze-drying, the vacuum was replaced with nitrogen gas and sealed with the tube still inserted in its port on the freeze-dryer. Non-dehisced pollen storage atmosphere changes from normal to nitrogen gas were accomplished by inserting cotton flowers collected before anthesis into 3 ml glass vials place inside a 25ml glass vial sealed with a 2-hole rubber stopper. Half the 3ml glass vials had water enough to cover the flower peduncle and half were without water. Nitrogen gas was flushed into the 25ml vials for 10 seconds, and each vial was sealed with corks in the holes of the rubber stopper and stored at 5°C. for 24, 48, 72, and 96 hours.

RESULTS AND DISCUSSION

The objective of this study was to seek ways of extending cotton pollen

longevity. Earlier studies resulted in procedures that extended the viability of cotton pollen from 48 to 72 hours with a household referigerator. In the summer of 1981 we found a significant decline in dehisced cotton pollen viability after 12 hours at 25°C. Holding the pollen at 5°C gave some improvement but all pollen was killed when held at −10°C. These data are shown in Table 1. The failure of storage at below freezing temperatures was unexpected. A subsequent experiment with pollen storage over calcium chloride (Table 2) gave a slight improvement when stored at 5°C. Again, freezing temperatures destroyed the viability of cotton pollen.

In vitro methods for evaluating the viability of cotton pollen were explored in the winter of 1980-81. TAYLOR's (15) germinating medium was modified by a series of single phase comparisons. One of these is shown in Table 3. TAYLOR's medium (loc. cit.) was 3.5% agar, 25% sucrose, 0.07% manganous sulfate, 0.04% calcium nitrate and 0.04% boric acid in distilled water adjusted to pH 7.0. As pointed out by BARROW (3) TAYLOR's average germination percentages and our results, shown in Table 3, confirmed the needed pH reported by TAYLOR (15).

A series of *in vitro* tests was conducted in the period from December, 1980 to February, 1982. These tests were made to evaluate the effects of varying pollen storage treatments. One factor examined was altered sucrose needs of cotton pollen stored after desiccation. LEOPOLD & KREIDEMAN (12) made this suggestion. Germination data shown in Table 4 indicated that the 15% sucrose used in the series of interim tests may have been a little low. In Table 4, the increased pollen germination after 3 hours of storage over $CaCl_2$ was assumed to be due to some desiccation or drying before the dehisced pollen was placed on the medium.

Interim tests (December, 1980 – February, 1982) were generally negative in extending cotton pollen longevity. Freezing in any form destroyed the viability of cotton pollen. Summaries of the interim tests are shown in Table 5.

Table 1

Effect of storage temperature on cotton pollen viability as measured by seeds per locule. All plants were field grown in 1980, pollen being collected 15 August.

Length of storage in hours	Storage temperature (C)			
	25°	5°	−10° 1/	Means 2/ of 25° and 5°C
		N.° seeds/locule		
4	4.6	6.0	0	5.30
8	3.4	5.1	0	4.25
12	2.8	3.2	0	3.00
24	0.7	0.7	0	1.55
Means	2.9	4.2*	0	
LSD. 05	For comparing time of storage means			1.03
30 1/	0	1.8	0	–
36 1/	0	0.5	0	–
48 1/	0	0	0	–
72 1/	0	0	0	–
96 1/	0	0	0	–

* Significantly different from 2.9
1/ Not included in the statistical analysis.
2/ The mean for fresh pollen was 6.2

Table 2

Cotton pollen viability as measured by seeds per locule affected by storage temperature and degree of calcium chloride desiccation. Pollen from field grown plants was collected 16 August, 1980.

Hours of storage over calcium chloride	Storage temperature (C)				
	25°	10°	5°	−10°[1/]	Mean[2/]
			N.° seeds/locule		
24	1.3	1.0	3.9	0	2.1
48	0.5	0.3	0.4	0	0.4
72[1/]	0	0	0.1	0	–
96[1/]	0	0	0	0	
Temperature means	0.9	0.6	1.5		
LSD.05	For comparing temperature means 0.76				

*Significantly different from 0.4
1/ Not included in the statistical analysis
2/ The mean for fresh pollen was 7.0

The largely negative results obtained in the series of interim tests raised some concerns of procedures. In the summer of 1982 both *in vivo* and *in vitro* evaluations were held at constat temperatures. These results are shown in Table 6. We found no apparent decline in effectiveness of evaluation procedures. There was an excellent agreement between *in vivo* and *in vitro* procedures.

We found that dehisced cotton pollen loses vigor rapidly, regardless of storage procedures. Freezing in any form kills cotton pollen. Flower removal prior to anthesis and held up to 72 hours at 5°C appeared to have no major decline in viability much as reported by HARRISON & FULTON (7) fifty years ago. We concluded that cotton pollen was unique in having a restricted storage potential using currently avaiable techniques.

Table 3

Comparison of fresh cotton pollen germination and tube growth at different pH levels for media used.

pH	Percent germination[1/]	Pollen tube length[2/]
	%	Um
4.0	6.5 e [3/]	177.5
4.5	12.0 d	272.5
5.0	9.5 de	260.0
5.4 (unadjusted)	50.0 c	537.5
6.0	45.0 c	487.5
7.0	78.7 a	737.5
7.5	63.2 b	642.5
8.0	60.7 b	697.5

1/ The plants from which flowers were taken were grown in greenhouse in the winter of 1980-1981. The pollen was collected 16 November, 1980.
2/ Linear response was highly significant; quadratic was significant
3/ pH level effects compared with Chi-square. Any two percentages followed by the same letter do not differ significantly at P = 0.01.

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Table 4

Effect of calcium chloride desiccation on cotton pollen germination with varying levels of sucrose in the media[1]

Length of storage over $CaCl_2$ at 5°C	Sucrose percentages[2/]			
	15	25	35	Average[3/]
(h) Hours		%		
0	42.2	31.2	3.4	25.6 b
3	34.0	57.9	0.0	30.6 a
6	30.6	30.7	0.0	20.5 c
10	23.9	29.8	2.1	18.6 c
12	7.1	10.4	1.3	6.3 d
24	0.0	1.4	0.9	0.8 e
36	1.6	0.7	0.0	0.8 e
Average[3/]	19.9 b	23.1 a	1.1 c	

1/ The plants from which flowers were taken were grown in greenhouse in the winter of 1981-1982. The pollen was collected 7 January, 1982.
2/ A medium with 45.0% sucrose was tested but none of the pollen germinated.
3/ Any two means followed by the same letter do not differ significantly according to an appropriate Chi-square test at P = 0.01.

Table 5

Summary statements of results from a series of tests conducted between December, 1980, and February, 1982 to extend cotton pollen longevity.

Treatments	Responses to:	
	w/prior desic.	*w.o./prior desic.*
(1) Freezing	negative 1	negative
	fast thaw	*slow thaw*
(2) Quick Freezing	negative	negative
(3) Vacuum drying	negative	negative
	w/moisture supplement	*w.o./moisture*
(4) Nitrogen gas	no gain[2]	no gain

1 A negative response indicates zero pollen germination at any level of storage.
2 A no gain response means that treatment had no significant advance at any duration of storage.

Table 6

Viability of cotton pollen estimated both *in vitro* and in vivo after being desiccated for varying periods over $CaCl_2$ (at 5°C)1/

Duration of prior storage over $CaCl_2$ at 5°C	*In vitro*		*in vivo*
	Germination2	Pollen tube length	Seed per locule
(h) Hours	%	Um	n.º
4	27.2 a 3	621.2	5.4
8	25.9 a	628.0	5.3
12	17.2 a b	525.6	1.8
24	20.7 b	586.0	3.5
36	3.4 c	240.8	0
48 4	0 –	0	0
LSD. 05		249.9	2.9

1/ The plants from which flowers were taken grown in the field during the summer of 1982. The pollen was collected on 2 August.

2/ The viability indices for fresh pollen before desiccation were: germination 46.0%, pollen tube length 648.8 um,and seed per locule 6.1.

3/ Germination percentages compared with Chi-square. Any two percentages followed by the same letter do not differ at P = 0.01.

4/ Not included in the statistical analysis.