Preliminary Evaluations of Techniques for Collecting and Culturing Peanut (**Arachis Hypogaea** L.) Pollen[1]

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ABSTRACT

The effects of time and method of pollen sampling, method and duration of pollen maintenance, media and plant genotype on peanut pollen germination and tube growth were observed. It was concluded that flower and anther sampling contributed much variability to pollen germination but the optimum times for pollen collection were from 3 a.m. to 8 a.m. Eastern Daylight Saving time during the summer months in Raleigh, N. C. Pollen maintained at 8 C germinated better and produced longer tubes than did pollen stored at room temperature. Desiccation of pollen for periods up to 6 days was found necessary if pollen was to be maintained more than 1 day. A 15% sucrose medium was found to maximize pollen germination. Attempts to differentiate parental and hybrid plant genotypes by pollen tube growth were not successful as flower to flower and plant pollen variability was too high to permit comparisons.

The peanut cultivars currently grown are derived from an extremely narrow genetic base (10). When one also considers the limited numbers of peanut breeders, the geographic separation of these breeders and the size and fragility of the peanut seed, the immediacy of the need for better methods of collecting, preserving, shipping and culturing of germ plasm becomes apparent. The investigation was designed to determine the effects of flower and anther sampling, method and duration of pollen maintenance, temperature of pollen maintenance, media sugar concentration and plant genotype on pollen germination (*in vitro*) and tube growth.

The peanut plant first flowers within 4 to 6 weeks after planting and continues to flower for approximately 4 months (10). The flower buds appear in the axils of foliage leaves or cataphylls and are first observed the afternoon preceding the day of flower opening (9, 17). The hypanthium or calyx tube is only 6-10 mm in length 24 hr prior to anthesis, but it elongates rapidly when night falls (16, 17). Barring inclement weather, the flower is open at daybreak (9, 16). In mature peanut flowers the anthers dehisce before or soon after anthesis causing self-pollination. Thus the space within the closed beak of the keel becomes packed with pollen grains (16).

Peanut pollen is said to have the highest viability when collected in early morning (4, 6). This finding has been corrobrated by *in vivo* (4, 17) and in vitro (2, 13) studies. Pollen collected as early as 3 a.m. was found to be physiologically immature, while that collected after 9 a.m. was over-mature (13). Since peanut pollen is very sensitive to changes in environment (2, 6, 9, 13), it is imperative that the investigator consider the light, temperature and humidity to which the plant is exposed.

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There is no single medium which will insure pollen germination (6, 8, 11, 19). The germination and elongation of the pollen tube are dependent upon water, oxygen and a suitable osmotic milieu (5, 11, 19). Very little is known about the physiology and biochemistry of the stigma exudates (11, 12) or of the substance sometimes called "pollenin" which Beck and Joly found to contain auxins (3). The age of the pollen may also influence germination as the oily substance on the grains tends to deteriorate with time (3). Other substances in the stigma said to have control of pollen tube elongation are known to be soluble in water, alcohol and ether and to be heat resistant (11).

A water-soluble growth factor that diffuses from the pollen grain into the medium tends to promote pollen germination (11). Pollen grains in groups, therefore, germinate more readily than those placed singly on the medium (5, 11). This phenomenon is commonly referred to as population effect (3, 11, 19).

Brewbaker and Kwack (5) proposed a medium that permitted good pollen germination and tube growth which resolved the pollen population effect. They used 86 species representing 39 families of higher plants. This medium contained 10% sucrose, 100 ppm H_3BO_3. 100 ppm KNO_3, 200 ppm $MgSO_4\ 7H_2O$ and 300 ppm $Ca(NO_3)_2\ 4H_2O$.

There are many methods of culturing pollen. Some investigators have placed pollen in a solution on a cover slip, which in turn, was placed on moist filter paper in a petri dish (5, 13). Others (2, 7, 13) preferred a solid or liquid medium on microscope slides but also held in a humid environment.

Materials and Methods

Peanut genotypes used in the evaluation of procedures, environments and/or media appropriate for pollen germination were derived from autogamous lines without previous hybridization. NC 4 is a progeny selection tracing to a single plant in 1929, while Va 56R comes from a single plant selection made in 1951 (1). The former is of bunch habit, while the latter is a runner. Both are classified as Virginia botanical types.

For a complete description of the parents and hybrids used in differentiating genotypes, the reader should refer to Parker et al. (15).

While methods, environments and media varied with specific investigations, the same basic procedures for collecting, maintaining, culturing and evaluating pollen germination and tube growth were used throughout the study.

Pollen was collected intact in open flowers (unless specified otherwise) with approximately 15-30mm of the hypanthium attached. Flowers were immediately placed in covered jars humidified by wet filter paper. Flowers not to be used for pollen within a period of 4 hr were maintained in covered jars under refrigeration at 8 C.

When pollen was desiccated, part of the corolla was removed, but the keel petal with its pollen intact was left attached to the hypanthium. The keel was placed in a small (1.3 cm x 1.8 cm) open-ended envelope which accommodated three or four keels. The envelopes were arranged in mini-desiccators which consisted of 5.2 cm x 3.4 cm screw-top bottles holding 4 g of $CaCl_2$ under a layer of absorbent cotton. If large quantities of pollen were to be desiccated, similar procedures were followed, but envelopes were held in screen-wire baskets (7 cm x 4 cm x 2 cm) and the latter stored in a pyrex desiccator. After desiccation, pollen (still in keel and in the envelope) was stored in clean dry bottles at desired temperature.

Regardless of the storage method, i.e., desiccation or otherwise, the pollen was kept in a warm and humid environment for a minimum of 1 hr prior to culturing. Wet filter paper was inserted into jars of stored pollen and these were set in pans of warm (30°C) water in preparation for culturing.

The basic medium used for culturing pollen was that described by Brewbaker and Kwack (5), but as a result of early experiments the concentration of $Ca(NO_3)_2$ was altered from 300 ppm to 350 ppm for all tests. Variations in sugar concentrations were assessed and the medium used for the final tests of stored pollen or of genotypes (Tables 3, 5, 7) contained 10.25% sucrose.

When solid medium was desired, 2 g of agar were added per 100 ml of liquid medium described above and the mixture heated to 100 C for its incorporation. The prepared media were autoclaved in laboratory dropper bottles and stored under refrigeration (8°C) until needed.

Ordinary microscope slides were used for both agar and liquid cultures. The routine procedures of culturing were as follows:

(a) Medium was dropped on the center of the slide

(b) With sterile forceps, the keel petal was opened and the stamens brushed lightly over agar smears or if liquid culture, the stamens were held tightly in the medium and the pollen allowed to diffuse from the anthers. The pollen in the keel was not separated from the pollen still in the anther except for comparison of pollen germination from specific anthers (Table 2).

Pollen germination was checked after 2 hr by simply counting three separate and randomly chosen fields, 100X (low power), of each slide. Pollen grains in each field varied in number from 6-250. Pollen was counted as germinated if the tubes were longer than the diameter of the pollen grain. Satisfactory germination was considered to be more than 10% even though some fields indicated 50% germination. The three shortest and three longest pollen tubes in each field were measured using a stage micrometer. The lengths were averaged for use in our experiments.

Results

The random sampling of flowers was found to contribute much variability to pollen germination. The flower to flower responses within a variety noted in Table 1 indicate that flowers #2 and #7, for example, had poor pollen germination regard-

Table 1. Germination of pollen within flowers* over sugar concentrations. †

Flower‡	Sucrose concentration of media (%)			
	5	10	15	20
1	+§	+	+	-
2	-	-	-	-
3	-	+	+	+
4	+	+	+	+
5	-	-	+	-
6	+	+	+	-
7	-	-	-	-
8	-	+	+	+
9	+	+	+	+
10	-	-	+	-

*Flowers collected randomly from plants.

†Pollen germination checked after 2 hr of culture.

‡One flower was used to culture four slides, one slide of each concentration of sucrose in medium.

§+ = more than 10% germination, - = less than 10% germination.

Table 2. Comparison of pollen germination from specific anthers within flowers* over sugar concentrations. †

Flower	Sucrose concentration of media (%) 5	10	15	20	Average
1	10.0‡	5.0	4.9	0.0	4.98
2	0.0	11.8	4.5	4.2	5.13
3	7.1	20.0	15.5	21.4	16.00

*Flowers collected at random.
†Percentage pollen germination checked after 2-hr culture period.
‡Germination determined on pollen from a single anther of same flower.

less of media, while others such as flowers #4 and #9 had satisfactory pollen germination in all media. Similarly in Table 2, differences were noted among the three flowers in mean pollen germination as well as among media when pollen from one anther was used per medium. Flower #3 had better germination than the other two flowers in three of the four media tested.

Viable pollen was collected for approximately 12 hr preceding and 12 hr after anthesis on both variety Va 56R and NC 4. On the latter variety, however, pollen germination dropped sharply approximately 5 hr after anthesis. Optimum times for collecting pollen appeared to be from 3 a.m. to 8 a.m. Eastern Daylight Saving time during the summer months in Raleigh (Table 3).

Once pollen has been collected as described in the Materials and Methods, it should be stored under refrigeration. When germination of pollen maintained at 8°C was compared with that maintained at room temperature, it was found that the mean percentage of satisfactory slides for pollen maintained at 8°C was 66.7, while those held at room temperatures produced only 45.3% satisfactory slides (Table 4). Secondly, the pollen tube growth was greater from that stored at 8° C than the pollen maintained at room temperature. Pollen germination generally decreased with increased days of maintenance, but this relationship was not constant.

Table 3. Germination of pollen from two genotypes collected over a 24-hr period.

Collection time	Va 56R Culture time	Va 56R Germination*	NC 4 Culture time	NC 4 Germination*
7 p.m.	8 a.m.	+	7:30 a.m.	+
8 p.m.	8 a.m.	++	7:30 a.m.	+
9 p.m.	8 a.m.	-	7:30 a.m.	+
10 p.m.	8 a.m.	+	7:30 a.m.	+
11 p.m.	8 a.m.	++	7:30 a.m.	+
12 midnight	8 a.m.	+	7:30 a.m.	-
1 a.m.	8 a.m.	+	7:30 a.m.	+
2 a.m.	8 a.m.	+	8:00 a.m.	
3 a.m.	8 a.m.	++	8:00 a.m.	++
4 a.m.	8 a.m.	++	8:00 a.m.	++
5 a.m.	8 a.m.	+++	8:00 a.m.	++
6 a.m.	8 a.m.	++	8:00 a.m.	+++
7 a.m.	8 a.m.	++	8:00 a.m.	++
8 a.m.	8 a.m.	++	8:00 a.m.	++
9 a.m.	9 a.m.	++	9:00 a.m.	+
10 a.m.	10 a.m.	++	10:00 a.m.	+
11 a.m.	11 a.m.	-	11:00 a.m.	-
12 noon	12 noon	++	12 noon	-
1 p.m.	1 p.m.	-	1:00 p.m.	-
2 p.m.	2 p.m.	+	2:00 p.m.	-
3 p.m.	3 p.m.	+	3:00 p.m.	-
4 p.m.	4 p.m.	+	4:00 p.m.	+
5 p.m.	5 p.m.	+	5:00 p.m.	-
6 p.m.	6 p.m.	+		

*0 = none, - = few, + = 10%, ++ = 30%, +++ = 50% germination after 2 hr culture on an average of three slides.
Three or more flowers checked each hour if results are given.

Table 4. The effect of duration and temperature of maintenance on pollen germination and tube lengths when cultured on sugar concentartions of 10-25% sucrose.*

Days stored	8 C # slides	8 C % sat. germ.†	8 C Range tube length (mm)	Room temperature‡ # slides	Room temperature‡ % sat. germ.†	Room temperature‡ Range tube length (mm)
1	12	91.7	31.8-80.0	12	58.3	14.5-67.8
2	12	83.3	17.8-68.5	12	33.3	12.0-63.3
3	12	58.3	17.5-65.3	11	27.3	19.3-35.5
4	11	54.5	17.8-73.8	11	63.4	11.3-54.0
5	11	45.5	21.5-61.8	9	44.4	13.3-56.8
Mean		66.7			45.3	

*Attempts were made to scan three slides per temperature per media per day of storage. Since there were four sucrose concentrations, the objective was 12 slides per day of storage.
†Satisfactory germination was considered to be 10%+.
‡Temperatures ranged from 25 to 33 C.

If pollen is to be maintained for more than a day, there is an advantage in desiccation. In trials using a 9-day storage period following varied periods of desiccation, satisfactory pollen germination was found following 6 days of desiccation (Table 5). One to 2 days of desiccation apparently is not adequate for the prevention of molds and germination dropped rapidly after 6 days of desiccation.

Pollen germination was found to be highest when cultured on a 15% sucrose medium (Table 6). This observation was particularly evident if the pollen had been maintained at room temperature and even when refrigerated, the percentage of satisfactory slides was high (Table 6). Pollen

Table 5. Pollen germination as influenced by desiccation at room temperature (25-33 C) and storage for 9 days at 8°C.*

No. flowers	Days desiccated	% satisfactory slides
18	1	0
17	2	0
18	6	100
19	7	52.6
16	8	18.8
18	9	16.7
17	10	5.9

*Pollen germination checked after 2 hr culture.

tube growth was found to be slightly greater in the 10% sucrose media than in the 15% concentration and poorest pollen tube growth occurred in the higher sugar concentrations (Table 6).

Table 6. The effect of maintenance temperature and sucrose concentration of media on pollen germination and tube length when maintained 1-5 days prior to culture.*

Sucrose conc (%)	8 C			Room temperature†		
	# slides	% sat. germ.	Range tube length (mm)	# slides	% sat. germ.	Range tube length (mm)
10	15	40.0	21.2-89.0	12	41.7	33.8-77.3
15	15	80.0	25.2-85.8	15	80.0	17.2-63.4
20	15	80.0	27.0-69.0	15	26.7	5.8-33.5
25	13	84.6	11.6-35.6	14	28.6	2.4-37.2

*Attempts were made to score three slides per temperature per media per day of storage. Since there were 5 days of maintenance, the objective was 15 slides per sucrose concentration. Germination was determined after 2 hr of culture.

†Temperatures ranged from 25-33 C.

Nine F_1 hybrids between homozygous peanut lines could not be differentiated by pollen germination when plants were grown in randomized complete block design. The replicate to replicate, slide to slide and microscope field to field variances prevented assessment of genotypes (Table 7). The obvious effects of technique and flower sampling indicated that statistical analyses were not warranted.

Table 7. Percentage peanut pollen germination as affected by sampling and genotype.

Genotype*	Slide 1† Scope Field 1	Slide 1† Scope Field 2	Avg germ./ Scope Field	Slide 2 Scope Field 1	Slide 2 Scope Field 2	Avg germ./ Scope Field	Avg germ./ slide/ rep
			Rep 1‡				
$A_2 \times C_2$	54.2	60.0	57.1	27.3	33.3	30.3	43.7
$B_1 \times C_2$	30.2	33.3	31.8	53.6	46.4	50.0	40.9
$C_2 \times C_2$	36.8	36.4	36.0	0	0	0	18.3
$B_1 \times A_2$	19.4	10.5	15.0	28.6	18.9	23.8	19.4
$A_1 \times A_2$	0	0	0	41.7	50.0	45.9	25.0
$A_1 \times C_2$	12.5	11.1	11.8	5.0	0	2.5	7.1
$A_2 \times B_2$	55.0	55.3	55.2	28.6	55.6	42.1	48.7
$C_1 \times B_1$	17.9	36.0	27.0	20.8	19.0	19.9	23.5
$C_1 \times C_2$	17.6	70.6	44.1	14.3	22.2	18.3	31.2
			Rep 2				
$A_2 \times C_2$	0	0	0	0	0	0	0
$B_1 \times C_2$	52.8	15.8	34.3	22.7	35.3	29.0	31.7
$C_2 \times C_2$	12.6	15.9	14.3	10.6	7.3	9.0	11.7
$B_1 \times A_2$	22.7	32.0	27.4	38.5	56.5	47.5	37.5
$A_1 \times A_2$	29.9	36.4	33.2	20.0	18.6	19.3	26.3
$A_1 \times C_2$	42.1	34.3	38.2	36.0	45.5	40.8	39.5
$A_2 \times B_2$	18.5	17.3	17.9	27.3	22.2	24.8	21.4
$C_1 \times B_1$	9.5	4.6	7.1	4.0	12.5	8.3	7.7
$C_1 \times C_2$	23.6	25.0	24.3	20.7	13.6	17.2	20.8

*A_1 = Plant Introduction 275751, a Valencia-type peanut from Peru, A_2 = A Valencia-type peanut from Peru, B_1 = Plant Introduction 262090, a Virginia-type peanut from Bolivia, B_2 = A Virginia-type peanut from Bolivia, C_1 = Plant Introduction 261924, a Spanish-type peanut from Argentina, and C_2 = Plant Introduction 262000, a Spanish-type peanut from Paraguay.

†Slide 1 = one flower, slide 2 = another flower.

‡Replicates are two groups of plants representing the same entries.

Discussion

Pollen germination was found to be extremely erratic in our investigation. While it is known that stress environments have adverse effects on peanut pollen (6, 10), the pollen used in our trials was derived from plants grown under greenhouse conditions and normal summer temperatures. Others (6, 18) have noted that pollen germination and tube lengths of collections from the same plant varied with the time of collection, but we found differential responses which could be directly attributed to flower variations on the same plant and within the same collection (Table 1). Both DeBeer (6) and Oakes (13) found significant differences in the viability of pollen which were caused by different stages of physiological development. These findings led us to conclude that large numbers of flowers should be collected over extended periods of time if the range of pollen maturities is to be properly sampled.

Should it be necessary to collect pollen at unusual times, *i.e.*, at times other than at anthesis, we found that viable pollen could be collected as early as 12 hr prior to and 12 hr after anthesis. In some varieties post-anthesis pollen may degenerate in less than 12 hr after anthesis (Table 3). Maximum viability was noted with pollen sampled from 3 a.m. to 8 a.m. Eastern Daylight Saving time when collected in July at Raleigh, N. C.

Humidity is critical to the life and germination of pollen, but specific requirements may vary with the species (11, 19). The methods used for maintaining pollen in our studies were designed to keep the pollen in its natural state, thus allowing the maturation process to continue during storage when it was not accomplished by the time of collection. Iwanami (11) points out that starch in the pollen grains disappears at inflorescence when "the pollen has finished its growth and each grain has become a free individual." The availability of sugar as an energy source in the pollen grain is necessary for the initial act of germination. We found high humidities and temperatures also essential to the germination of peanut pollen.

If pollen is to be maintained over days, care must be taken to prevent the growth of fungi (3). To prevent the deterioration of pollen, it was desiccated prior to storage at 8°C. Poor success was found when periods of desiccation were for less than 3 days, presumably due to inadequate drying and increased growth of fungi. Periods of 6 to 10 days of desiccation followed by storage in dry containers at 8°C were found quite satisfactory for short-term maintenance of pollen (Table 5).

After evaluations of media for culturing, we found the Brewbaker and Kwack formulation (5) with 10% sucrose gave maximum tube growth and minimal bursting while 15% sucrose concentration maximized the pollen germination (Table 6). Results of early tests involving each of the nutrients used in the Brewbaker and Kwack formulation (5) caused us to alter only two ingredients: sucrose from 10% to 10.25% and Ca-$(NO_3)_2$ from 300 ppm to 350 ppm. We adjusted

the pH of the medium to 5.5 in keeping with Brewbaker and Kwack. We have found no record of optimum pH for peanut pollen growth.

The improvised germination chamber provided a constant warm, humid environment for a maximum of 20 slides. The dome-like top of the bread box prevented the condensation of moisture on the media.

The wide differences in pollen germination from collections made from the same plant or within the same flower did not allow accurate evaluations of peanut genotypes. We, therefore, could not determine whether parental and hybrid pollen germinations could be differentiated by tube lengths.

With increasing interest in the development and preservation of germplasm, the techniques used in these experiments to collect, preserve and culture peanut pollen must be refined. However, it was clearly demonstrated that pollen desiccated for 6 to 7 days and stored for 9 days at 8 C was still viable and gave good germination (Table 5). Further work (unpublished) on long-term storage of peanut pollen is encouraging and the authors are continuing these investigations in an attempt to perfect a method to store peanut pollen for great lengths of time.

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