STUDIES TO AID PEANUT BREEDING TECHNIQUES

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CHAPTER I

INTRODUCTION

The peanut is recognized as one of the important legume crops of the world. Though the peanut is a tropical crop, its cultivation extends over vast areas of the tropical, sub-tropical, and temperate regions.

Superior peanut varieties are being developed by employing various plant breeding techniques, with hybridization considered to be the more promising method for improvement. Large numbers of hybrids are required to provide reasonable chances of obtaining superior agronomic types. However, because of the peculiar habit of the peanut, the number of hybrids which can be obtained is limited by the available crossing techniques.

Peanut flowers are borne on inflorescences in the leaf axils, either above or below the ground. In a mature flower the stigma usually lies buried among the dehisced anthers in the tightly closed keel. The anthers ripen during the night and usually dehisce before dawn (14). The gynophore or peg, which developes into a fruit, is an outgrowth of the ovary, and is positively geotropic. Peg growth begins immediately after fertilization. Each peg bears two to six ovules near the tip, depending on the variety. Pegs more than 15 cm. above the soil surface usually fail to reach the ground and the fruit do not develop.

Each successful pollination usually provides no more than two seeds.

The tedious hybridization technique requires much time and exact timing for performing these operations is critical. The percentage of hand cross pollinations which result in fertilizations varies widely, ranging from 1 to 75 percent (29). Thus, the production of individual hybrids is comparatively low. Improvements in breeding techniques could greatly aid the peanut improvement program. The purpose of these studies was to obtain information to improve peanut breeding techniques. Hybridization techniques, pollen fertility, and methods of peg culture were studied.

CHAPTER II

REVIEW OF LITERATURE

The earliest peanut breeding work was reported by Van der Stok in 1910 (32). He made plant selections in several local and introduced varieties. He stated that the structure of the peanut flower necessitates self-pollination and that natural outcrosses occur rarely. We know now that some natural crossing by wild bees takes place (8). Van der Stok's artificial cross-pollination technique consisted of two separate operations: removal of the intact anther sacs from the flower buds (emasculation) during the late evening of the day preceding blooming and application of pollen from a male parent (pollination) early the following morning. Some modified cross-pollination techniques and different percentages of fertilization in peanuts have been reported by several other peanut breeders (1, 29, 31, 24). Environmental conditions such as humidity and temperature were indicated as being important factors which influenced the percentage of successful fertilizations (25, 10, 27). Schultz (27) reported that placing the plants in a simple moist chamber immediately after pollination, effectively increased fertilization from 62 to 84 percent. His moist chamber consisted of moistened cheesecloth constructed on a greenhouse bench.

The viability of pollen is an important factor when hybridizating peanuts. Important factors influencing the germination of pollen include water, sugar, boron, and calcium. An analysis of the stigmatic

extract of Nymphaea by Schmucker /according to Vasil (33)/ showed appreciable quantities of boric acid. He found that the addition of boric acid to the pollen culture medium not only improved germination, it also stimulated elongation of the pollen tubes. In a series of biochemical studies on pollen and plant extracts and on conventional growth constituents, Brewbaker and Kwack (5) found that calcium is an important growth promoting constituent of the pollen tube. Autoradiographic studies of pollen tubes grown in the presence of calcium 45 revealed that calcium was taken up almost exclusively in or on the pollen wall (4).

Oakes (22) found 8 to 10 percent sucrose to be optimum for the germination of peanut pollen. Later, Vasil (33) found that pollen germination percentage and tube lengths could be highly increased by adding 0.01 percent boric acid.

Peanut pollen viability studies have been made by several workers (22, 10, 33, 16). Oakes (22) found significant differences in pollen viability when pollen collections were made at different stages of physiological development. Pollen viability increased from 3:00 to 5:00 a.m., remained about the same from 5:00 to 7:00 a.m., and gradually decreased from 7:00 to 9:00 a.m. De Beer (10) used 8 percent sucrose with 1 percent shredded agar as a pollen culture medium. He found that the pollen germination percentages and tube lengths of pollen collected from the same plant in a greenhouse varied considerably throughout the day. In his study, the pollen germination decreased from 35 percent 15 minutes after daybreak to 3 percent, 10 hours later. The maximum lengths of the pollen tubes were 1660μ when germination took place shortly after daybreak, and 996μ, 498μ, 83μ, when germination occurred

4, 7, or 10 hours later, respectively. His study indicated that pollen germination and tube lengths were high at the beginning of anthesis and decreased rapidly with time. Jorhi and Vasil (18) reported that maximum peanut pollen germination was 27 percent in 10 percent sucrose-agar with 0.01 percent boric acid. One hour and 55 minutes after sowing the pollen grains, the pollen tubes had reached a maximum length of 4680μ.

The viability of pollen of most plants decreases in storage. Oakes (22) found that the viability of peanut pollen collected at 7:00 a.m. could be retained for eight days and that collected at 3:00 a.m., 5:00 a.m., and 9:00 a.m. for six days, when whole flowers were stored in open vials placed in desiccators over calcium chloride at 45° F. Ballaux (2) reported that peanut pollen could be stored by placing the flower on a watch-glass in a refrigerator at a temperature of 2° C. and a relative humidity of 45 to 50 percent for more than 40 days with only a gradual loss in viability.

The time and duration that stigmas are receptive to pollen are factors that plant breeders must consider.

Hassan and Srivastava (16) reported that peanut stigmas were receptive to pollen from 24 hours before to 12 hours after the flowers opened.

The study of pollen germination on stigmas and tube growth in the styles of plants have been important in determining cross compatibilities within and among varieties and species. Various techniques have been used for these studies which involve the staining of microtome sections and squashes. Some of the methods that have been successful on other plants are as follows: Acid fuchsin-light green-with pectinase on Physalis (23) and without pectinase on Datura (6); Lacmoid-martius

yellow in pomaceous plants (21); Acetocarmine-magenta for the pistils of plants with central canals (7); Safranin O and aniline blue on Solanum and several other genera (11); Lactophenol-cotton blue was reported to be successful on 36 plant species (9). The fluorescence method of staining pollen tubes in tomato styles by Martin (20) depends on the occurrence of callose, a polysaccharide, which lines and plugs pollen grains and pollen tubes. Callose in living or dead tissue can be stained selectively with water soluble aniline blue and similar dyes which fluoresce in ultraviolet light. Martin's technique is summarized as follows: Dissected entire styles were fixed for 24 hours in FAA then cleared and softened in a strong ($\pm$ 8 N) sodium hydroxide solution. Staining was accomplished in a 0.1 percent solution of water soluble aniline blue dye dissolved in 0.1N potassium hypophosphate. The styles were mounted in a few drops of the staining medium on clean slides and cover slips were applied. Observations were made by ultraviolet microscopy with an approximate wave length of 356 mμ. Under these conditions the callose in the pollen tubes fluoresces bright yellow to yellow-green, whereas the background tissue fluoresces pale gray or blue. Oakes (22) used paraffin sections cut with a rotary microtome to study pollen tube growth in peanut styles. The sections were stained in 1 percent acid fuchsin and mounted in Canada balsam.

In the study of chromosome number and morphology, treatment with chemicals such as colchicine and acenaphthene are usually employed to accumulate metaphase figures and to aid in chromosome separation. In a study of chromosome morphology, Storey and Mann (30) introduced a selective herbicide IPC (o-Isopropyl-N-Phenlycarbamate) which caused contraction of chromosomes in the prophase, metaphase, and anaphase

stages of mitosis. They found IPC to be effective on the chromosomes of root tips of several species which included a member of the leguminosae family. However, no work has been reported on using IPC to contract the chromosomes during mitotic divisions of pollen in vitro.

A peculiar feature of peanut plants is the fruits that are produced underground by pegs which grow into the soil. Hassan and Srivastova (16) found that under natural conditions, the pegs were visible from 3 to 7 days and entered the soil 5 to 8 days after the flowers opened. The pegs began to swell 10 to 20 days after blooming. On the 40th day, the fleshiness of the pods decreased considerably at the basal end but were still fleshy at the apical end. An air-space developed between the seed (ovule) and the shell and faint pigmentation enveloped the entire seed by the 50th day after flowering. The early varieties were mature on the 60th day after blooming.

Little work has been done on trying to culture peanut pegs. Harris (15) achieved some success in growing individual pods in glass test tubes by wrapping the pegs with cheesecloth or glass cloth soaked in distilled water saturated with calcium sulfate and 0.2 ppm of boron. The wrapped pegs were inserted into the tube containing 2 ml. of distilled water and light was excluded with aluminum foil.

CHAPTER III

MATERIALS AND METHODS

Hybridization Techniques

Seeds of Argentine, Starr, Dixie Spanish, and "Krinkle" Spanish were planted in forty 10-inch clay pots. Twenty pots of each consisting of 5 pots for each variety, were grown in a greenhouse bench and in a growth chamber in a randomized design. Each pot contained one plant.

The growth chamber conditions consisted of a 12 hour day (9:00 a.m. -9:00 p.m.) with 82° F. and 76% relative humidity and a 12 hour night (9:00 p.m. - 9:00 a.m.) with 68° F. and 66% relative humidity.

Temperature in the greenhouse was not accurately controlled but generally ranged around 80° - 85° F. during the day and night. Humidity was not controlled and was variable.

Hybridization studies were conducted both in the greenhouse and growth chamber. The "Krinkle" Spanish variety was used as the pollen parent while Argentine, Starr, and Dixie Spanish varieties were used as female parents. By using the dominant mutant "krinkle" character, hybrids resulting from the pollinations were easily detected as seedlings.

The following crossing techniques were tried:

1. Conventional pollination method

   A modified Van der Stok method of crossing was used. All of the flowers were removed from the inflorescences except the

ones to be hand pollinated. The flowers were emasculated between 7:00 and 9:00 p.m. by removing the anthers with forceps. Pollinations were made the following morning between 6:30 and 8:00 a.m. in the greenhouse, and between 9:00 and 10:00 a.m. in the growth chamber. The cross-pollinated flowers were labelled with small tags attached by a thread. These tags were later transferred to the pegs.

2. Clipped style method

Instead of removing the anthers individually with forceps, parts of the flowers were cut with a sharp knife and removed. The style stumps were pollinated the same day between 6:30 and 7:30 a.m. in the greenhouse, and 9:00 to 10:00 a.m. in the growth chamber. These flowers were labelled in the same way as in the conventional method.

Four positions (Fig. 1) for cutting of the flower were chosen and pollinations were made with and without the addition of a pollen culture medium to the style stumps.

The pollen culture medium containing 8 percent sucrose with 1 percent agar and 0.01 percent boron was applied as a small drop with a pipette on the cut surface of the styles.

Emerging pegs one week after pollination were noted and recorded. The putative hybrid seeds were planted after harvesting the mature pods. The seedlings which showed krinkle leaflets, indicated successes and were recorded.

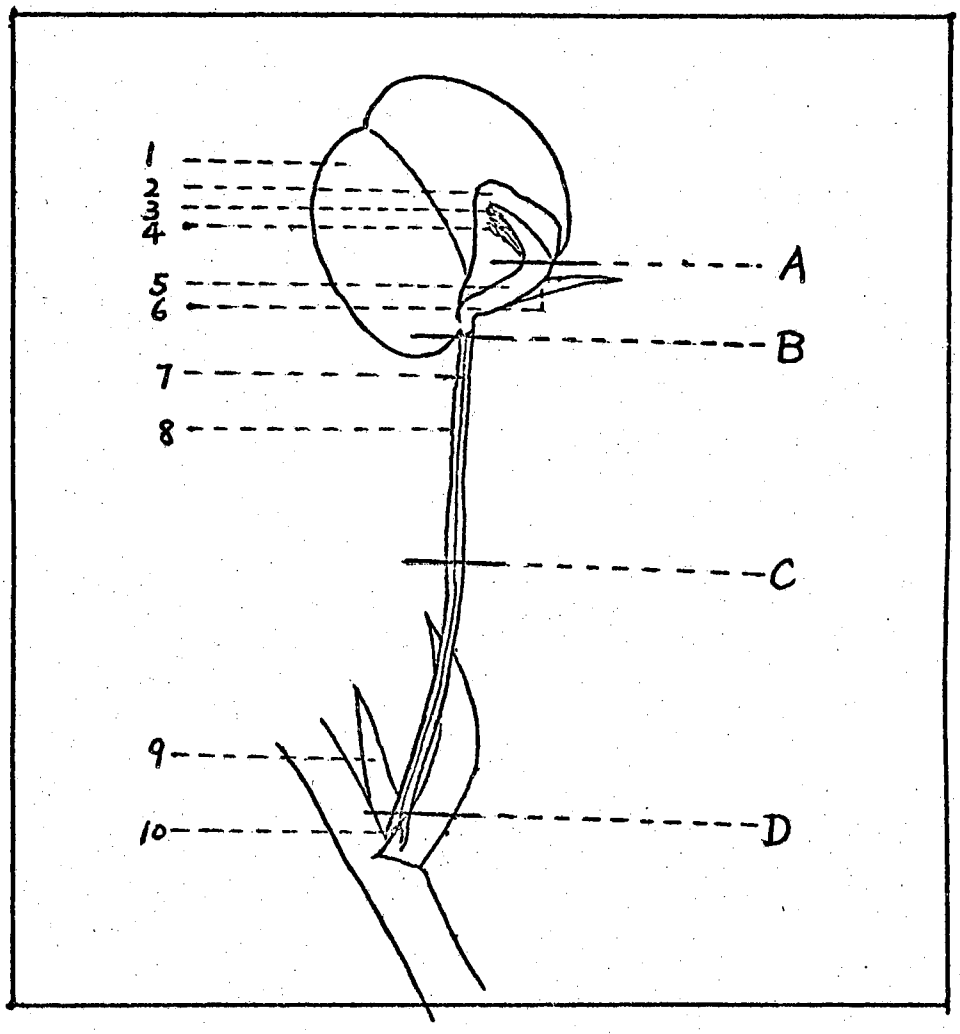


Figure 1. A Longitudinal View of a Peanut Flower. 1. standard; 2. wing; 3. stigma; 4. anthers; 5. keel; 6. calyx; 7. style; 8. hypanthium (calyx tube); 9. bracts; 10. ovary.
A. clipped at the fusion point of the staminal column;
B. clipped under the coralla;
C. clipped at the mid length of the calyx tube;
D. clipped at the base of the calyx tube.

Pollen Fertility Studies

1. Staining pollen tubes within the styles

Several published methods and modifications for studying pollen tubes in the styles were attempted. These methods included Acid fuchsin-light green-with pectinase, Lacmoid-martius yellow, Acetocarmine, Acetocarmine-magenta, Lacmoid, Safranin O-aniline blue, Lactophenol-cotton blue, Iodine, Acid fuchsin-light green, and Martin's fluorescent method.

The peanut pistils studied by Martin's fluorescence method were collected in the greenhouse around 3:00 to 4:00 p.m. and placed immediately in a killing solution composed of 1 part formalin, 8 parts 80 percent alcohol and 1 part glacial acetic acid. Pistils were left in this solution for 24 hours or more. Afterward they were rinsed in tap water and softened 8 hours in 8 N sodium hydroxide. The styles were dissected from the pistils. The rest of the procedure followed Martin (20) as previously described.

Observations were made with a Zeiss Fluorescence Microscope employing a UG-5 Exciter filter and 41 and -65 barrier filters using a dark field condensor and a 250 HBO mercury burner. The Ug-5 filter removes wave lengths between 420 and 680 mμ. The combination 41 and -65 filters pass this spectrum.

2. Pollen tube cultures

In an effort to germinate peanut pollen, the following culture media were tried: 10 percent sucrose with 0.01 percent Boron both with and without agar, or with gelatin, and

the medium described by Brewbaker. Brewbaker's medium consisted of 10 percent sucrose, 100 ppm boric acid, 300 ppm calcium nitrate, 200 ppm magnesium sulfate, 100 ppm potassium nitrate dissolved in deionized water. For the study of mitosis in the cultured pollen tubes, 0.1 percent colchicine, and 50, 100, and 200 ppm of IPC were added to Brewbaker's medium. Acenaphthene were also tried.

Pollen germination and tube growth studies were made by the following methods: the hanging drop method, millipore membrane method (17), collodion membrance method (26), and the floating cellophane method. Squares of cellophane (2 X 2 cm) were wet in the above media and the excess was removed by blotting. Squares were floated on a large drop of the media in petri dishes. Pollen collected in the greenhouse from 7:00 to 8:00 a.m. was dusted on the exposed surface of the cellophane. Petri dish lids were lined with wet blotting paper to form moist germination chambers. The cultures were incubated at room temperature on a table top under ordinary room illuminating. The pollen was allowed to germinate for at least 2 hours before counts were made to determine germinating percentage. Pollen tube lengths were measured with an ocular micrometer. The ten longest tubes were measured and recorded. Cytological studies of mitotic divisions were made on the pollen grains that were fixed at hourly intervals during a 12 hour period. Final fixation was made 24 hours after culturing. For studying nuclear divisions in pollen grains, microscopic observations were made by transferring the squares onto slides.

Fixation was accomplished by adding one to two drops of acetic-alcohol (1:3) on the squares. Pollen grains and tubes on the squares were stained with acetocarmine and covered with a cover slip.

Peg Culture Studies

Attempts were made to culture pegs both in vitro and on the plants. Methods for culturing were as follows:

1. In vitro

   A. Sections approximately 2 cm long were cut from the tip of young pegs on plants which were used for the crossing experiments in the greenhouse. The pegs were sterilized by soaking for 3 minutes in 1 percent chlorox, rinsed in sterilized distilled water and transferred under a ultraviolet light hood to autoclaved petri dishes, or test tubes, containing a sterile modified White's medium (12). The containers were then placed in a dark cabinet at room temperature.

   One peg each in thirty vials and two pegs each in twenty petri dishes were cultured. The pegs were observed periodically to ascertain if growth took place. Final observations were made after 45 days.

   B. Sections approximately 4 - 7 cm long from young pegs were cut from the plants and sterilized as above. The cut ends of sterilized pegs were placed in vials containing sterile modified White's medium by inserting them through a hole in the center of a cork. The corks were sealed aseptically to prevent contamination. The operation was conducted under a

ultraviolet light hood. The tips of the pegs were covered with soil by supporting the vials on a rack.

Twenty-four pegs were cultured. Final examination was made after 60 days (Fig. 2).

2. Culturing pegs on plants

Pegs were inserted into small polyethylene bags containing either soil, peat, or a mixture of peat-perlite (1:1). Preliminary trials had indicated that darkness was necessary to stop peg elongation (Fig. 3). Therefore the bags were wrapped with aluminum foil to exclude light and were tied with wire to seal the top. The growing media were moistened periodically by pouring water through a drinking straw which was inserted in the bag (Fig. 4).

Pegs were examined after 28 days to determine if fruits had developed.

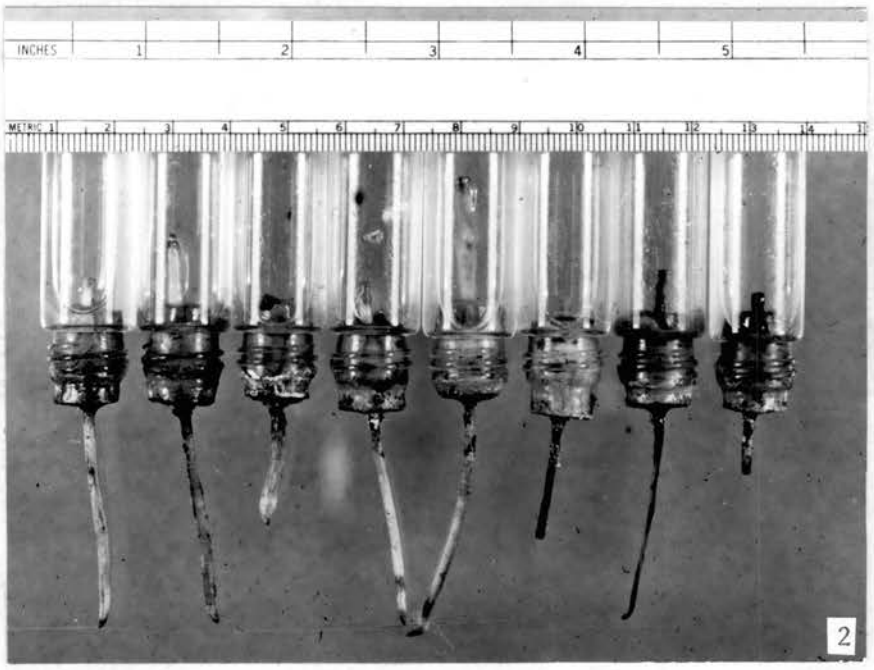


Figure 2. Method of Insection of Pegs in Bottles with Modified White's Medium. (Pegs had failed to grow after one and a half months.)

Figure 3. Peg Cultured in Soil in a Small Plastic Bag Without Excluding the Light. (Note the peg growing through the plastic bag.)

Figure 4. Pegs Cultured in Soil Medium on a Plant.

CHAPTER IV

RESULTS AND DISCUSSION

Hybridization Techniques

Results of the cross-pollination studies are shown in Table I. The percentages of fertilizations were calculated by dividing the number of flowers by the number of pegs. Thirteen F_1 krinkle-leaf plants from the growth chamber test and two from the greenhouse test were obtained from 30 putative hybrid seedlings. During the test many tags were lost because the strings had rotted, therefore, the percent hybrids obtained was much less than the number of pegs produced. Since the number of opening flowers was variable and because the time in conducting emasculations and pollinations was limited, it was impossible to make the same numbers of crosses on a given day and under both conditions. Therefore, the analysis of variance depended on the success values of percentage of fertilization (Table II). The F value shows that there was no significant difference in the results obtained under the two environmental conditions. However, the percentage of fertilization was more variable in the greenhouse than in the growth chamber. Exact reasons for the differences are not known but differences in temperature, humidity, and light between the two conditions are probably involved. De Beer reported that the later in the day that pollen sampling took place, the lower the pollen viability, the higher the temperature during illumination, the quicker the viability decreased, and the behaviour of

TABLE I

RESULTS OF FERTILIZATION IN THE GREENHOUSE AND GROWTH CHAMBER

Date of Crossing	Greenhouse Conditions			Growth Chamber		
	Pollinations Attempted	Fertilizations	Percent Fertilization	Pollinations Attempted	Fertilizations	Percent Fertilization
July 11	14	8	57.1	48	18	37.5
July 28	14	5	35.7	19	6	31.6
August 11	30	3	10.0	53	23	43.4
August 18	12	2	16.7	26	12	46.2
Wt. Average			25.7			40.4

pollen was mainly dependent on temperature conditions 36 to 96 hours preceding the actual opening of the flowers. Fortanier (13) reported that peanut flowers open a few minutes after illumination begins, but the time of opening depends also on the illumination that the peanut plants were exposed to three days previously. Similar results were obtained in these experiments. After the plants were moved from the greenhouse to the growth chamber, the time of opening of the flowers changed from the normal opening time around 6:00 a.m. to immediately after 9:00 a.m. three days later.

TABLE II

ANALYSIS OF VARIANCE FOR PERCENTAGE OF FERTILIZATION IN A COMPARISON OF GREENHOUSE AND GROWTH CHAMBER CONDITION

Analysis of Variance				
	d.f.	S.S.	M.S.	F.
Total	7	1664.43575		
Treatment	1	191.2968	191.2968	0.77913
Error	6	1473.13895	245.52315	

F.05 = 5.99

The results of the clipped-style pollination method conducted under both conditions with several treatments are shown in Table III. No pegs were produced from any of these treatments. These results suggest that the clipped-style method will have little value in peanut hybridization techniques.

The stigmatic cells were noted to secrete appreciable amounts of an oily fluid which probably stimulates pollen germination and growth

TABLE III

RESULTS OF HYBRIDIZATION ATTEMPTS BY USING CLIPPED STYLE METHOD ON FOUR POSITIONS OF THE FLOWER WITH AND WITHOUT CULTURE MEDIA ON THE STYLE STUMPS

Date of Crossing	Treatment	Greenhouse Condition Pollinations Attempted	Growth Chamber Pollinations Attempted
July 10	A (Without Medium)	5	6
	B (" ")	15	8
	C (" ")	5	5
	D (" ")	10	6
July 12	A (" ")	5	5
	B (" ")	5	5
	C (" ")	5	5
	D (" ")	5	5
July 13	A (" ")	10	3
	B (" ")	9	5
	C (" ")	9	8
	D (" ")	5	5
July 14	A (With Medium)	8	9
	B (" ")	10	9
	C (" ")	6	12
	D (" ")	8	6
July 16	A (" ")	10	9
	B (" ")	10	12
	C (" ")	7	4
	D (" ")	12	3

in the style (28).

The failure to achieve pegs following the clipped-style pollination technique might be due to the lack of a specific stimulation due to the removal of the stigma even though a pollen culture medium was applied on the exposed surface of the resulting stump.

Because the stigma of the peanut flowers, like any legume, is protectively enclosed in the keel, the stumps resulting from clipping the style were exposed to atmospheric conditions. Dehydration of the stumps was observed. Thus, pollen germination may have been retarded. The anatomy of the peanut flower is such that measures to prevent this condition were considered to be impractical. Microscopic examinations of the stumps were not made to ascertain if pollen germination did occur. Future investigations should look at this aspect.

Pollen Fertility Studies

1. Staining pollen tubes within the style

   All of the staining methods tried allowed for the observation of the pollen tubes only on the stigmatic surface or for a very short distance within the stigmatic branch (Fig. 5). Since the stylar tissues were also stained, these methods were inadequate for tracing the pollen tubes. The pollen tubes could be identified only by their small diameter and the absence of cross walls. Figure 6 shows that the upper portion of the pollen tube became indistinguishable in the stylar tissue. It was difficult to stain the pollen tubes in the region surrounded by epidermal hairs near the tip of the stigma, except by the fluorescence method. The fluorescence method gave

Figure 5. Stigma and Style Stained in Acid Fuchsin-Light Green. (Note the visibility of pollen tubes only a very short distance from the end of the stigma.)

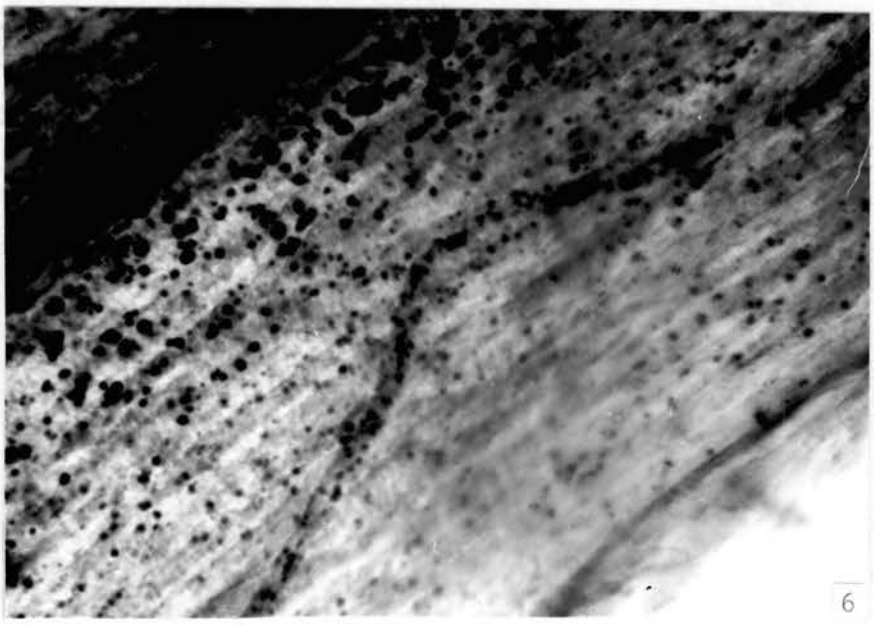


Figure 6. Stigma and Style Stained in Iodine. (Shows that the upper portion of the pollen tube is obscured in the style tissue.)

good results. The bright yellow callose in the pollen tubes were found to be in contrast to the blue background (Fig. 7). Because the pollen tubes are lined with and have irregularly spaced collose plugs, the entire lengths of the pollen tubes were visible in the styles. However, the intensity of fluorescence under the ultraviolet light decreased with time within a few minutes, so it was difficult to photograph or observe for a long period of time.

2. Growing pollen tubes in culture

In this study, 33 percent germination was obtained with the medium containing 10 percent sucrose with 0.01 percent boric acid with the longest tube length being 1010u. Low germination and short tube length were nearly always noticed in the other media except Brewbaker's. Of the various techniques, dusting pollen onto the fluid drop of the medium was the easiest method, but the pollen sank and poor germination often occurred. In order to avoid this problem,1 percent agar was added to the medium but agar increased the difficulty of staining the pollen tubes. Peanut pollen did not germinate well on the millipore membrane with the above agar medium. Savage's collodion membrane technique was unsuccessful because the membrane formed was somewhat corrugated and pollen sank in the medium, decreasing the total pollen germination. The Brewbaker's medium and the floating cellophane method produced significantly higher germination of peanut pollen than other media and methods tried.

The percentages of germination are shown in Table IV. The

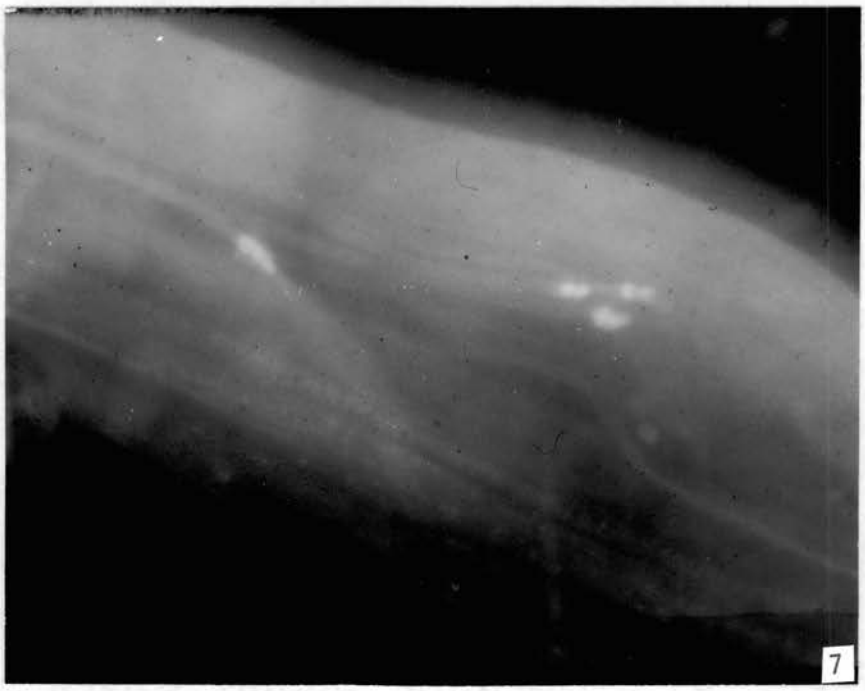


Figure 7. Pollen Tubes in the Style Stained by Fluorescence Method. (Staining was accomplished in a 0.1% solution of water soluble aniline blue dye dissolved in 0.1 N potassium hypophosphate.)

average of the ten longest pollen tubes measured was 4465μ. It was noticed that the pollen tubes were still growing after 10 hours except for a few tubes which had burst. Some of the pollen tubes were still growing after 12 hours but all growth had ceased by 24 hours. Within 5 hours culturing, the generative nucleus had divided and the division increased with time. After 6 hours, division of the generative nucleus had occurred in the pollen grains whether they had germinated or not. Fig. 8 shows two divided sperm cells prior to moving into the pollen tube 9 hours after culturing. After 12 hours (Fig. 9) many mitotic divisions had occurred in the tubes and pollen grains. After 24 hours, nuclear division could be seen in almost all the tubes and grains.

TABLE IV

PERCENTAGES OF GERMINATION OF POLLEN GRAINS ON THE BREWBAKER MEDIUM

Rep.	Total no. pollen grains counted	No. pollen grains germinated	Germination percentage
1	454	376	82.8
2	628	498	79.3
3	548	368	67.1
4	624	483	77.4
5	438	347	79.2
Wt. Avg.			76.96

The use of colchicine and acenaphthene in the Brewbaker medium delayed or inhibited nuclear divisions in the pollen

Figure 8. Pollen Grains After Culturing for 9 Hours on the Brewbaker Medium. (Note the 2 sperm nuclei moving to the tube in the center grain. Stained in acetocarmine.)

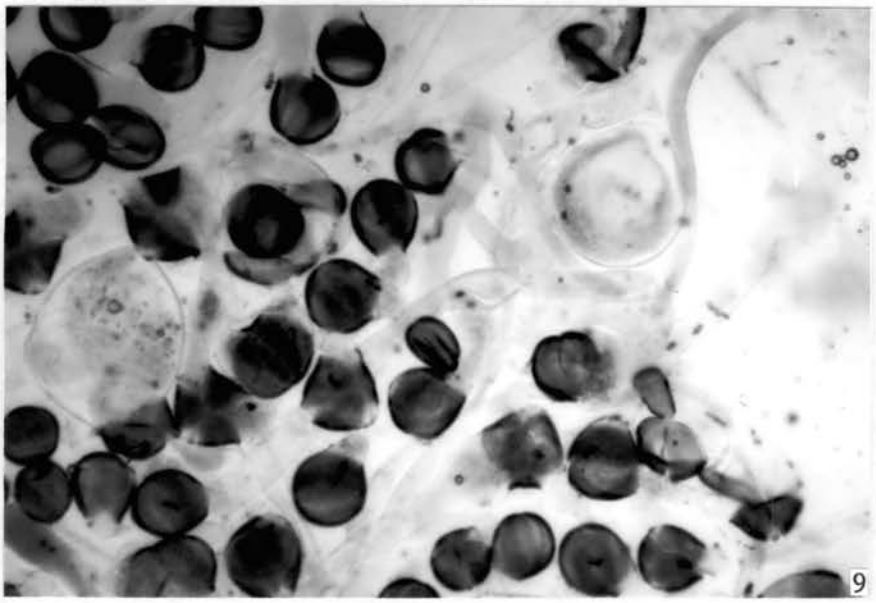
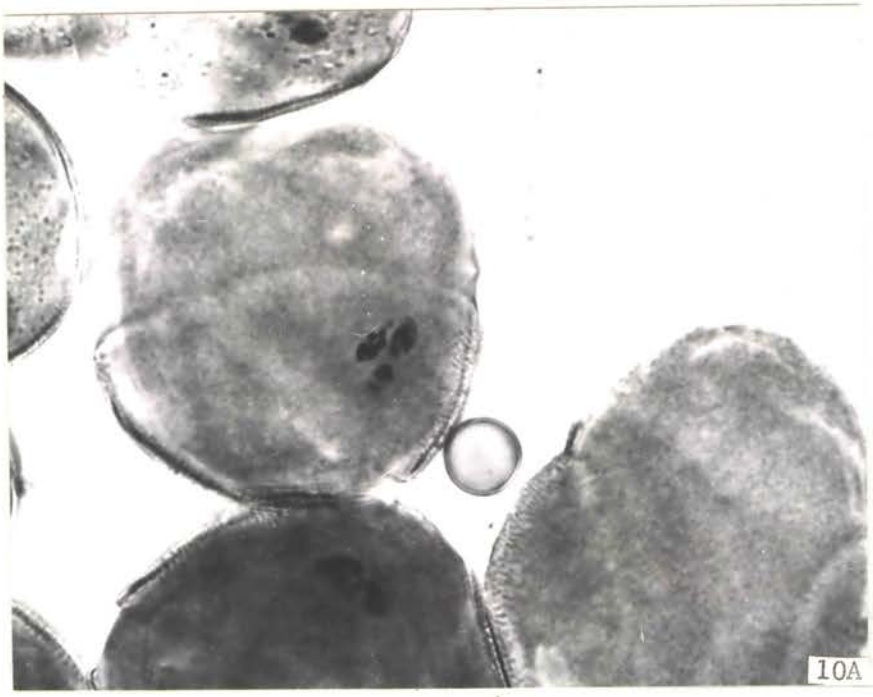


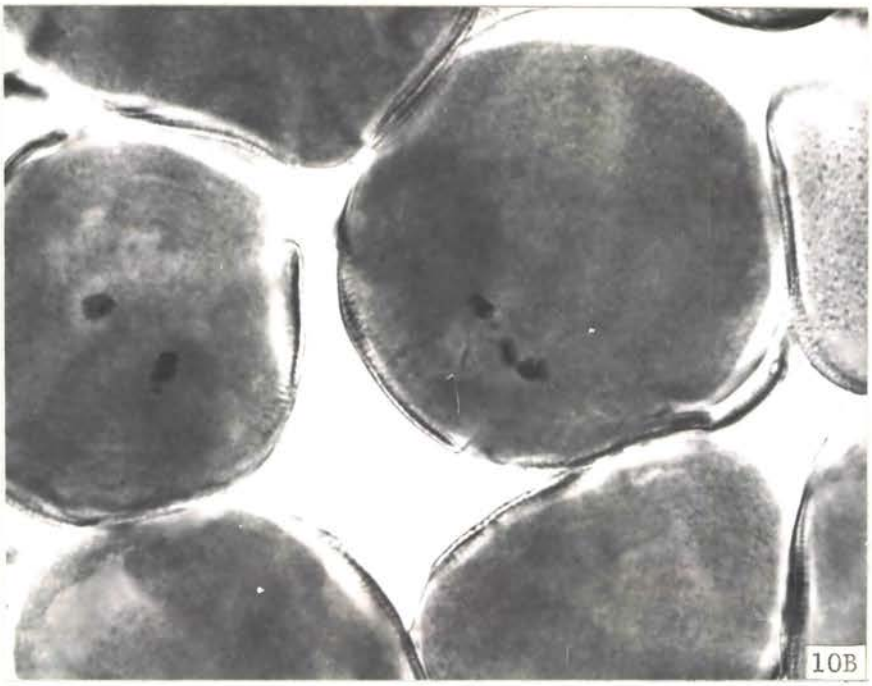
Figure 9. Pollen Grains After Culturing for 12 Hours on the Brewbaker Medium. (Note the 2 sperm nuclei in many of the pollen tubes and pollen grains. Stained in acetocarmine.)

grains and tubes. When 50 ppm of IPC was added to the medium, nuclear division occurred after 5 hours of culturing. By 24 hours, almost all of the generative nuclei had divided. Most of the divisions occurred in the pollen grains. Some irregular mitotic divisions were noted. These irregularities included the formation of 3 sperm nuclei (Fig. 10A, 10B) and micronuclei (Fig. 10C). The addition of 100 ppm of IPC to the medium, caused divisions to occur in both the pollen tubes and pollen grains (Fig. 11A) and 3 nuclei were observed in several grains (Fig. 11B). Figure 12 shows 2 sperm nuclei and the vegetative nucleus 12 hours after treatment. In most cases the vegetative nucleus was very difficult to stain. The presence of 3 sperm nuclei in pollen tubes were not observed. When 200 ppm IPC was added to the medium, the germination percentage and nuclear divisions were inhibited. Irregular divisions were not found in pollen grains with this medium.

Satisfactory preparations for studying peanut chromosomes in pollen grains and tubes were not achieved in this study. The dividing nuclei in all cases showed clumped chromosome and the determination of their number and morphology was impossible. According to Brewbaker (3) peanut pollen grains are binucleate and contain a generative and a vegetative or pollen tube nucleus. The generative cell undergoes mitosis during pollen tube growth and two sperm cells formed which take part in double fertilization in the embryo sac. There are no reports in the literature that sperm formation in peanuts occurs in the pollen grains. The formation of three sperm



10A



10B

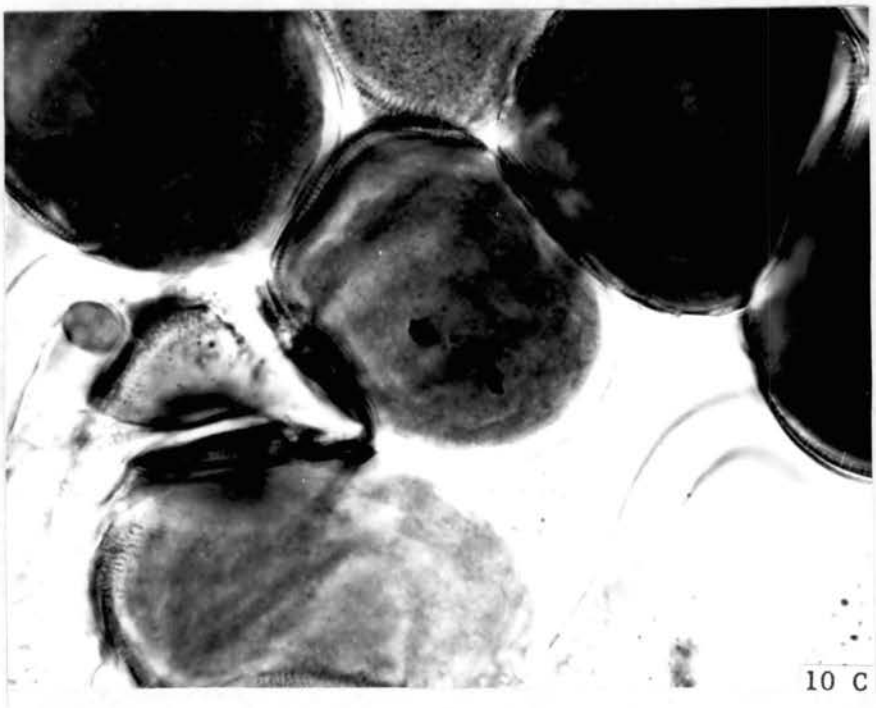


Figure 10. Pollen Grains After Culturing for 24 Hours on the Brewbaker Medium with 50 ppm IPC. (Stained in acetocarmine.
A. Note the 3 sperm nuclei.
B. Note the micronuclei.
C. Note the micronucleus.)

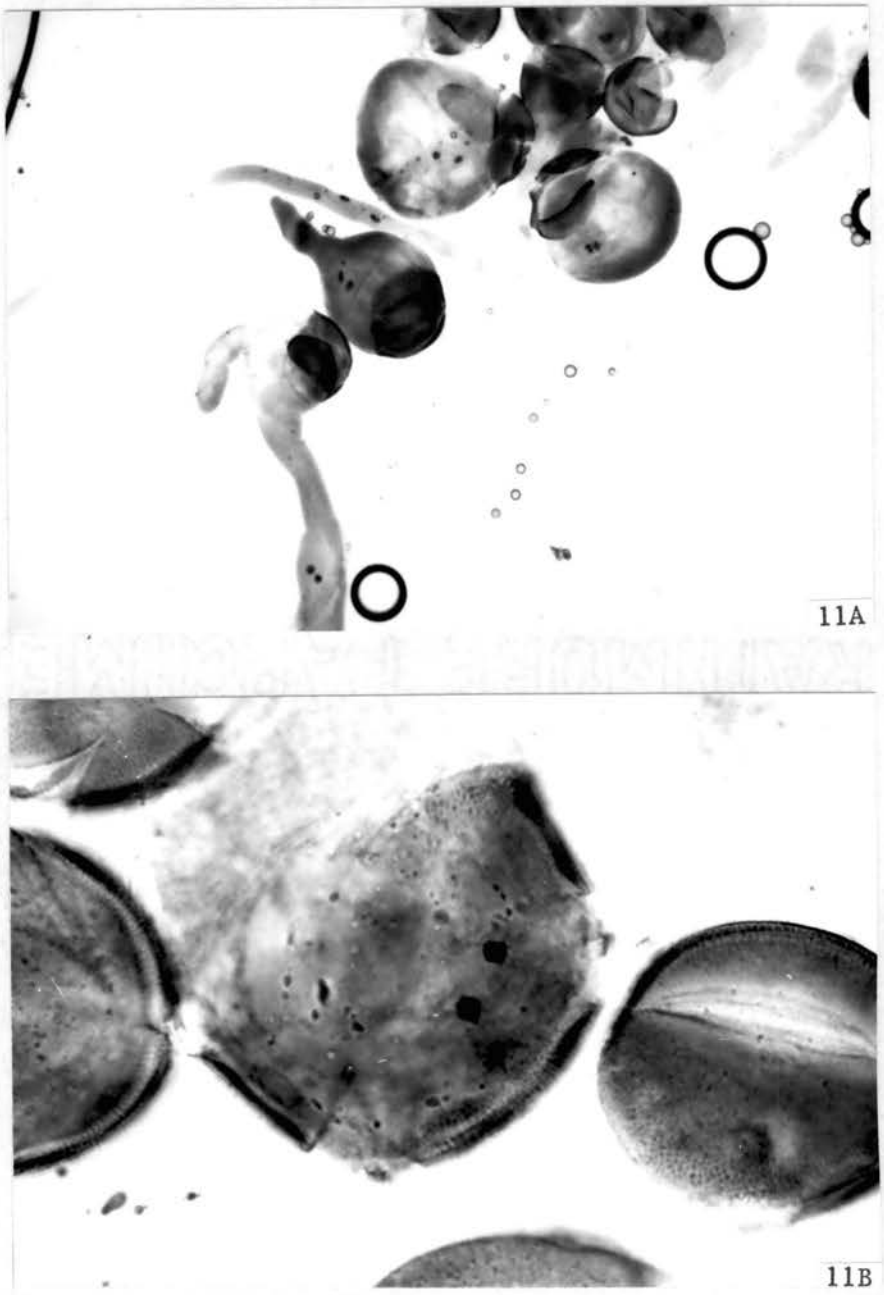


Figure 11. Pollen Grains After Culturing for 24 Hours on the Brewbaker Medium with 100 ppm IPC.
(A. Shows the division of the generative nucleus occurring in pollen tubes and pollen grains.
B. Note the 3 sperm nuclei. Stained in acetocarmine.)

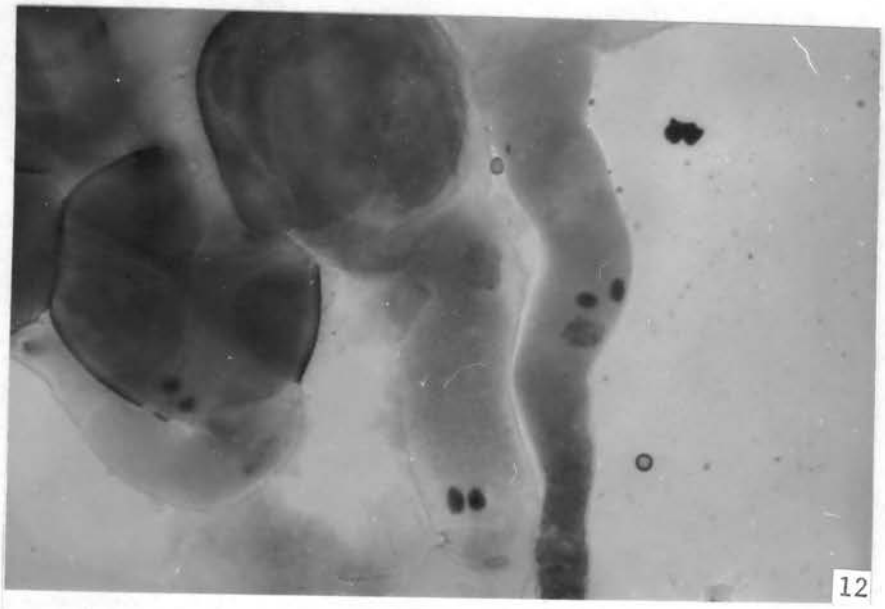


Figure 12. Pollen Grains 12 Hours After Culturing on the Brewbaker Medium with 100 ppm IPC. (Note the 2 sperm nuclei and vegetative nucleus. Stained in acetocarmine.)

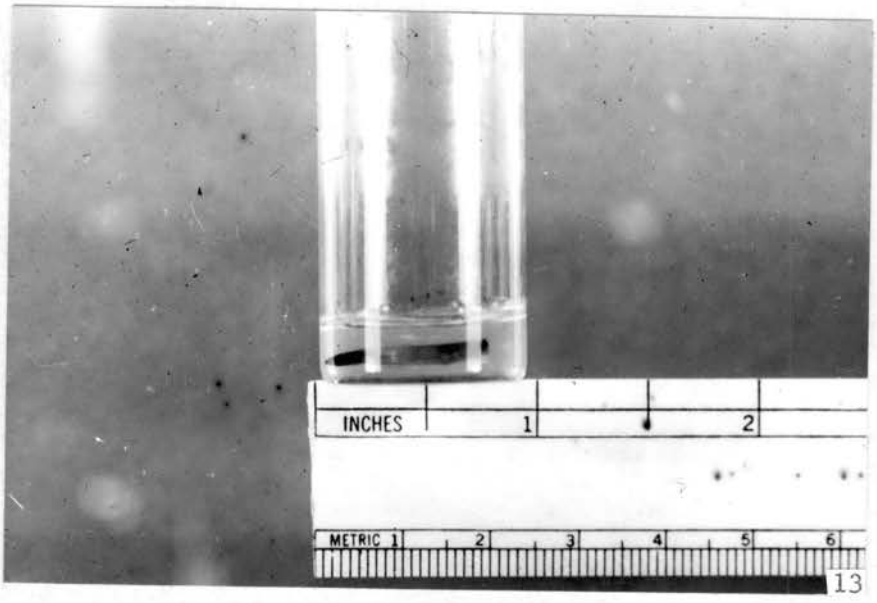


Figure 13. A Peanut Peg Cultured in a Modified White's Medium for 53 Days. (No growth of the peg was apparent.)

nuclei in the pollen grains in this study is abnormal. Because the chromosomes were not distinct, their number in the grains and tubes could not be determined. Irregular mitotic divisions occurred only when IPC was added to the culture medium. IPC is an analogue of phenylurethane, and is one of a small number of similar compounds used as selective herbicides. This compound was reported to be effective in causing contraction and separation of chromosomes in aceto-carmine and aceto-orcein squashes. The results here indicate that it can produce chromosomal abberations.

Peg Culture Studies

Both methods tried for growing the pegs in vitro (Fig. 13 and Fig. 2) failed to develop fruit. The failure of fruits to develop may have been due either to the lack of proper constituents in the medium or to poor conduction of the medium into the peg. Perhaps smaller amounts of tissue should have been tried. Bacteria and fungi contamination were also a problem.

Results of a comparison of the three different media on peg culture on the plants are shown in Table V. Pods developed satisfactory in all of the media tried. Although the soil medium showed the highest percent of success, the analysis of variance (Table VI) did not indicate any significant difference between treatments at the 5 percent level of significance.

TABLE V

A COMPARISON OF THE THREE DIFFERENT MEDIA FOR PEG CULTURE IN VIVO

Treatment / Date	Soil			Peat			Peat-Perlite (1:1)		
	No. Attempts	No. Pods Formed	Percent Success	No. Attempts	No. Pods Formed	Percent Success	No. Attempts	No. Pods Formed	Percent Success
Dec. 14, 1967	6	6	100	6	6	100	6	5	83
Dec. 26, 1968	12	10	83	12	9	75	12	8	66
Wt. Avg.			88			83			72

TABLE VI

ANALYSIS OF VARIANCE FOR THE PERCENTAGE OF THE SUCCESS ON DIFFERENT MEDIA

Analysis of Variance				
	d.f.	S.S.	M.S.	F.
Total	5	891.1019		
Treatment	2	300.8796	150.4398	0.76466
Error	3	590.2223	196.74076	

$F_{.05} = 9.55$

CHAPTER V

SUMMARY

During this study attempts were made to develop and improve techniques which could be used by plant breeder to aid peanut hybridization programs. The following results were obtained.

1. Hybridization techniques
   A. The conventional method of emasculation and pollination was the most successful one tried.
   B. The technique of clipping the flower at various levels to remove the anther and upper parts of the stigma and style followed by pollinating the resulting style stump with and without the addition of artificial pollen culture media was completely unsuccessful.

Further investigations are still needed to improve the crossing technique.

2. Pollen fertility studies
   A. None of the squash stain methods employed gave very satisfactory results except for the fluorescence method.
   B. The best culture medium tried for germinating peanut pollen and growing pollen tubes consisted of 10 percent sucrose, 100 ppm boric acid, 300 ppm calcium nitrate, 200 ppm magnesium sulfate, and 100 ppm potassium nitrate dissolved in deionized water. The

maximum pollen tube length obtained with this medium was 6256μ, and the mean germination was 77 percent.

The pollen tubes could be grown in the medium for more than 12 hours.

Five hours after germination on the medium the generative nucleus divided to form sperm cells in the pollen grains and tubes.

C. Colchicine and acenaphthene were ineffective in accumulating metaphase figures in cultured pollen tubes and grains.

D. Irregular divisions of the generative nuclei occurred when 50 and 100 ppm IPC was added to the culture medium. Further studies of IPC should be made to test its possible mutagenic properties.

E. In no cases did any of the treatments produce chromosomes that were clearly distinct so as to enable counting or study of their morphology in pollen tubes or grains.

3. Peg culture

A. Peanut pegs failed to develop properly when cultured in vitro using a modified White's medium.

B. Pods developed normally when soil, peat and peat-perlite mixtures were applied to the pegs by small plastic bags. This technique may be useful to peanut breeders.

Further studies are needed to develop better and more efficient methods for obtaining larger numbers of peanut hybrids.

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