SOME ANATOMICAL, HISTOCHEMICAL AND BIOCHEMICAL ANALYSES OF ENDOSPERM IN THE GENUS ARACHIS

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PREFACE

The following pages, dealing with the morphology and composition of peanut endosperm, are somewhat preliminary in nature but offer a great deal of "food for thought" to a wide range of scientists including research investigators in Agronomy, Biochemistry and Botany.

This investigation was encouraged and supervised by my major professor Dr. Donald J. Banks. I wish to extend my sincere thanks to Dr. Banks for his valuable advise and assistance in preparation of this thesis and suggestions concerning the techniques employed throughout the course of this study. The knowledge and leadership Dr. Banks shared with me during my undergraduate and graduate training have been an inspiration both academically and personally. Grateful appreciation is also extended to the other members of my advisory committee, Dr. J. M. Davidson, Department of Agronomy, and Dr. R. W. McNew, Department of Mathematics and Statistics for their assistance in interpretation of statistical data and offering constructive suggestions in preparation of this thesis.

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ABBREVIATIONS

Asp	aspartic acid
Thr	threonine
Ser	serine
Asn	asparagine
Glu	glutamic acid
Gln	glutamine
Pro	proline
Gly	glycine
Ala	alanine
α-H_2N But	alpha aminobutyric acid
Val	valine
Met	methionine
Ile	isoleucine
Leu	leucine
Phe	phenylalanine
His	histidine
Arg	arginine
NH_4	ammonia
Unk	unknown
μ	micron
$\mu\ell$	microliter

CHAPTER I

INTRODUCTION

The cultivated peanut, Arachis hypogaea L., along with its wild relatives, Arachis sp., belong to the tribe Hedysareae of the family Papilionaceae (1). Mature seeds of these leguminous plants are generally considered to be ex-albuminous (i.e. without endosperm). In many plants, for example, members of the Gramineae, Euphorbiaceae, and Solanaceae, the endosperm stores food substances which are utilized by the germinating seed. Generally, however, the endosperm is depleted in the Leguminosae by the growing embryo prior to seed maturation and the cotyledons serve as the primary food storage tissues for the germinating seed.

The endosperm, like the embryo, is dependent on fertilization and it is subject to similar genetic influences. Phenotypic differences between plants of contrasting genotypes are often obvious, thus it is plausible that phenotypic differences in endosperm might be detectable in some stages of development in diverse peanut germ plasm. Because the endosperm is a nutritive tissue, the expected differences might be quantitative or qualitative in regard to the chemical components of the endosperm. Differences in these components could be controlled generally and "genetic markers" might be present.

Few investigations concerning the ontogeny of the endosperm of cultivated peanuts have been made (12, 32, 34, 40, 41). There are

several reasons why such studies would be valuable to peanut breeders. Interspecific hybridizations with the wild species appear to be essential for the acquisition of some important agronomic characters, including genetic resistance to variance diseases and pests, for improving cultivated peanuts (3). However, embryo abortion following certain interspecific hybridizations prevents peanut breeders from making progress by this method. It appears likely that these abortions are, in part, due to irregular endosperm behavior (8). Appropriate measures might be developed for utilizing these wild species in breeding programs if the mechanisms governing successful interspecific hybridizations were better understood. Therefore, any information concerning the composition and characteristics of the endosperm might prove to be useful.

This study is divided into two sections.

1. ENDOSPERM MORPHOLOGY

The objective was to determine the nature and extent of the endosperm of peanuts as the ovules developed and to clarify some contradictions in the literature concerning the presence of endosperm in the mature seed (12, 32). The determination of the influence of endosperm in relation to the developing embryo in certain hybrid combinations (wild x cultivated) was also of serious concern. The methods used in this study involved microscopic examination of the developing embryos and endosperms from interspecific hybrids (made by reciprocal pollinations) and self-pollinated plants. This subject is discussed in Chapter II.

2. ENDOSPERM COMPOSITION

Several studies were initiated concerning the physical (amount, form or morphology) and chemical characteristics of some of the

important constituents of peanut endosperm. These studies were aimed at detecting possible "endosperm markers" or the determination of genetic differences that might be useful to the peanut breeder in identifying hybrids or for distinguishing between different genotypes.

Histochemical tests and thin-layer chromatography were employed to identify some of the components of the endosperm in order to better understand the role that the endosperm plays in embryo development. This chemical approach might clarify some of the causes of embryo abortion. Fluorometric measurements of lipase activity in the endosperm and studies of starch granules using x-ray diffraction, size distribution data, and birefringence end-point temperatures (BEPT) were used in an attempt to detect differences in these components among several genotypes. The studies dealing with endosperm composition are described in Chapters III-VIII. The last chapter is based on a paper presented before the Oklahoma Academy of Science (29).

General Materials and Methods

Thirteen peanut genotypes representing diversity between species and subspecies[a] were used in these studies. Their phenotypic descriptions and other pertinent information are given in Table I. Mention is made in each chapter of the ovule sizes and genotypes used for the respective studies.

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[a]Sub-species as used here refers to any taxonomic category below species.

TABLE I

PHENOTYPIC DESCRIPTIONS OF THE PEANUT GENOTYPES USED IN THE ENDOSPERM STUDIES

Genotype[a]	Name and/or P.I. No.	Species[b]	Botanical[c] Type	Seed Coat Color	Other[d] Characteristics	Plant[e] Location
P-112	Spanhoma	hypo.	S	Flesh	Typical Spanish	Pk, GH, GC
P-161	Tenn. Red	hypo.	S (Val)	Red	Typical Valencia	Pk
P-204	NC4x	hypo.	V	Flesh	Related to NC4 used by Conagin (12)	Pk
P-326	Guanajuato-2 P.I. 280688	hypo.	V	Purple	Purple pigmentation in stem, leaf, and flower	Pk
P-935	Mani Pintar II P.I. 268837	hypo.	V	Red and White Mottled	Typical Bunch Virginia	Pk
P-936	P.I. 262129	hypo.	S (Val)	Flesh with Purple Streaks	Large leaves, thick stems with glandular hairs on stipules	Pk
P-1284	Aureus	hypo.	S	Flesh	Golden color leaves	Pk
P-1286	Narrowleaflet	hypo.	S	Flesh	Dwarf plant with very narrow leaflets	Pk
P-1540	P.I. 262133	?	wild	Flesh	Yellow flowers, 2n=20 wide interspecific crossability	Pk,GH,GC
P-1562	P.I. 219823	duranensis*	wild	Flesh	Wide interspecific crossability	FC
P-1563	P.I. 210553	monticola	wild	Flesh	Produces fertile hybrids with hypo.	FC
P-2341	TMV-1	hypo.	V	Flesh	Used by Prakash (32)	Pk
P-2395	Nambyquare	hypo.	V	Purple and White Mottled	Large seed, prostrate habit	Pk

[a] P-numbers assigned by the Oklahoma Agricultural Experiment Station.

[b] hypo. = hypogaea (cultivated peanut)
? = un-named species
* = According to Smartt (37)

[c] S = Spanish, Val = Valencia; V = Virginia.

[d] All plants had orange flowers and were tetraploid (2n=40) unless otherwise indicated.

[e] Pk = Agronomy Research Station, Perkins, Oklahoma.
GH = Greenhouse, Stillwater, Oklahoma.
GC = Growth Chamber, Stillwater, Oklahoma.
FC = Caddo Peanut Research Station, Fort Cobb, Oklahoma.

Seeds were planted in early June at the rate of 3-4 seeds per foot (Pk and FC) and one seed per pot (GH and GC). Plants were harvested in late October, except for the studies in Chapters III and IV where they were harvested when needed throughout the growing season.

CHAPTER II

ENDOSPERM DEVELOPMENT IN OVULES OF INTERSPECIFIC HYBRIDS AND HAND POLLINATED 'SELFS' IN THE GENUS ARACHIS

Introduction

Information concerning the ontogeny of peanut endosperm is limited. The earliest work, according to Prakash (32), was Guignard's in 1882. Later Reed (34), in 1924, described the embryogeny of the peanut in a detailed study but his work was documented by rather poor terminology and illustrations. More detailed and informative work was reported by Smith (41), Conagin (12), and Prakash (32).

Smith (41) undertook a comprehensive study of the formation of the embryo sac and the early development of the embryo and endosperm (up to 10 days after pollination). Conagin (12) and Prakash (32) studied the development of the endosperm of the peanut following fertilization but they disagreed as to its presence in the mature seed. Conagin indicated that endosperm disappears during seed maturation but Prakash reported it was present as a two celled layer, "gorged with fatty food reserves," in mature seeds.

Previous authors referred to the early developing endosperm as 'free-nuclear' or 'nuclear endosperm.' In this study the term non-cellular will be used rather than the former terms because the latter is more appropriate according to Rao (33). The endosperm of the peanut

develops, following fertilization, by rapidly dividing nuclei. At first, the nuclei are free (i.e. without cell walls). Later, however, wall formation occurs beginning at the micropylar end of the ovule and continuing toward the chalazal region.

Failure of the endosperm, resulting in failure of an otherwise normal embryo, occurs frequently following interspecific hybridization between plants with different chromosome numbers (5, 7, 14, 26). Boyes and Thompson (7), after attempting reciprocal interspecific hybrids between Triticum and Secale, stated, "Whatever may be the primary cause of poor seeds and lack of success in crossing, it expresses itself through the endosperm."

Apparently, endosperm plays a major role in the development and maintenance of a medium of growth substances for the young embryo (46). Consequently, the death of the embryo following interspecific hybridization may not necessarily be a function of the embryo itself but it may be caused by an abnormal development of the endosperm.

Several suggestions have been made as to the cause of seed failure in interspecific hybrids (5, 8, 9, 13). These failures are probably due to chromosomal, genic, or cytoplasmic imbalances within the endosperm or incompatibility between the embryo and adjacent tissues (e.g. antipodals or nucellus). Johansen and Smith (26) reported seed failure following interspecific hybridization in peanuts. They made studies of aborting ovules by anatomical observations but could not ascribe the primary causes of failure to either the embryo or endosperm.

Smartt and Gregory (38), in their Arachis species cross

compatibility studies, discovered that a wild diploid annual species (10038)[a] would cross widely with other wild species and with A. hypogaea. However, successful crosses with A. hypogaea and this wild species were obtained only when the latter was used as the female parent. Although the hybrids were triploid and sterile their results are of interest because successful crosses, where plants of different ploidy levels are involved, usually occur when the female parent possesses the higher chromosome number (44).

The purpose of this investigation was to clarify the contradictions in the literature concerning the presence of endosperm in mature seeds; and to follow the development of the embryo and endosperm in hand pollinated 'selfs' and interspecific crosses between A. hypogaea and A. sp. (P-1540), which produce viable seeds only when the latter is the female parent. It was hoped that comparisons of endosperm behavior between normally developing seeds and aborting seeds would offer a better understanding of the nature of the endosperm's role as a medium for the growing embryo.

Materials and Methods

Two genotypes, P-112 (2n = 40) and P-1540 (2n = 20), were selected for the interspecific hybridization study. P-112 is A. hypogaea, a cultivated peanut. P-1540 is the wild un-named species. Six plants of P-112 and four plants of P-1540 were grown in eight inch and 14 inch plastic pots, respectively, containing a 1:1:1 mixture of peat, perlite and soil. Environmental conditions were controlled by growing the

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[a]This species is the same as P-1540 that was used in this study.

plants in a growth chamber on a 12 hour day schedule with the day period beginning at 6:00 pm and ending at 6:00 am. The day and night temperatures were 28 C and 22 C, respectively. Crosses were made reciprocally between these parents using each as egg and pollen sources. In addition, hand pollinated selfs were made so that comparisons would be possible. Emasculations were made between 7:30 and 9:30 am on flowers that would open during the day period. The anthers dehise and the pollen is shed at noon or later in flowers on plants grown under the growth chamber conditions described. Pollinations were made immediately using pollen collected from plants grown in pots in the greenhouse. The flowers were tagged, just prior to pollination, to indicate the pollination date and pollen source. The tags were wired to the developing pegs when they reached a length of 5 to 8 mm. Thus, the age and parents were known for each developing pod.

The number of pollinations, pegs formed, and ovaries, pegs, or ovules collected for anatomical observations are recorded below (the female parent is listed first):

Crosses	Pollinations	Pegs Formed	Ovaries, Pegs, or Ovules Collected
P-1540 x P-1540	114	85	80
P-1540 x P-112	124	111	90
P-112 x P-112	53	50	50
P-112 x P-1540	130	112	100

Ovaries, pegs, or ovules (depending on the stage of development) from the crosses and self pollinations were collected at the time of pollination and at two hour intervals from eight hours to 24 hours after pollination and 1, 2, 3, 4, 7, 21, 28, 42, and 60 days after pollination.

For the study on the extent, presence and amount of endosperm in

mature seeds of A. hypogaea, P-204 and P-2341 were used in addition to P-112. These genotypes were chosen because P-2341 (TMV-1) is the same genotype that Prakash (32) used and P-204 (NC4x) is closely related to NC4, the genotype that Conagin (12) used in her study. Because the above investigators had disagreed as to the presence of endosperm in mature seeds, ovules from these genotypes were collected only when completely mature as indicated by the dark endocarp of the pod. The pods were collected from plants grown in the field (see Table I).

In all studies the ovaries, pegs, or ovules were fixed in Craf, dehydrated in tertiary butyl alcohol and embedded in paraffin (Paraplast). The 42 day and older ovules were pretreated by soaking them in acetone for 24 hours prior to dehydration to remove the excess oil so they could be properly infiltrated and embedded. The sections were cut serially on a rotary microtome at 10 or 20 μ, depending on the size of the ovule, stained with Johansen's safranin and fast green (27), and mounted on slides with Canada balsam. Observations of the slides were made with a Zeiss GFL microscope and microphotographs were made of the significant sections on High Contrast Copy film.

Results and Discussion

Normal Endosperm Development

Even though observations were made of sections prior to and immediately following fertilizations, little reference will be made to them, since a more comprehensive study was undertaken by Smith (41) on ovules up to 10 days after pollination. Early observations made in this study were solely for the purpose of recognizing the developing endosperm so that its presence in the mature seed might be positively

identified.

Observations of fertilization and early development of the embryo and endosperm were in agreement with those noted by Smith (40, 41). The endosperm is a rapidly dividing tissue of a non-cellular state, which is oriented along the periphery of the embryonic cavity and surrounds the developing embryo (Figure 1). Wall formation begins to occur at the micropylar region in ovules of 3-4 mm in length at approximately 21 days of age. In later stages of development the endosperm is entirely cellular and is continually reduced in thickness (Figure 2). The observed sections in which the cotyledons had filled the embryonic cavity showed several layers of cellular endosperm at various degrees of thickness. These observations suggested that the endosperm nourishes the developing embryo and is consumed by it.

Throughout its development, the endosperm appeared to line the inside of the two cotyledons (Figure 3). The endosperm also appeared to be present in a mature seed as a single layer of cells between the cotyledons and seed coat (Figure 4) and between the two cotyledons (similar to that shown in Figure 3).

The presence and location of cellular endosperm in seeds collected at maturity of P-204 and P-2341 were similar to those shown in the previous figures (i.e. a single layer of endosperm lining the embryo at maturity).

Hybrid Endosperm Development

Fertilization occurred normally in the hybrid ovules regardless of which species (wild or cultivated) was used as the female. However, when compared to the hand pollinated selfs, the development of the

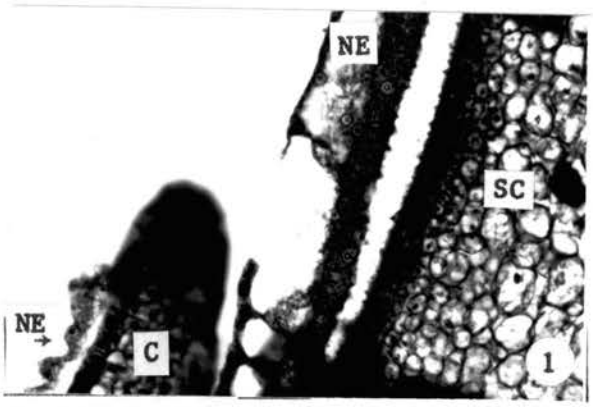
Figures 1-4. Various Stages of Endosperm Development.

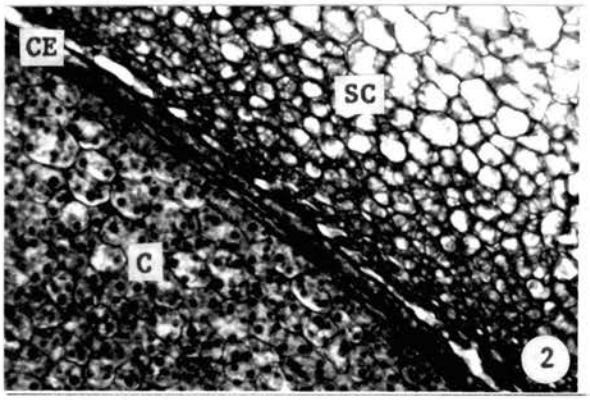
Figure 1. At 21 days, non-cellular endosperm (NE) covering the embryo (C) and becoming cellular along the periphery of the embryonic cavity (EC).

Figure 2. At 42 days, three layers of cellular endosperm (CE) between the cotyledon and seed coat (SC).

Figure 3. Cellular endosperm between both cotyledon halves at 42 days.

Figure 4. Cellular endosperm present at maturity as a single layer of cells between the cotyledon and seed coat.


NE
SC
NE
C
1


CE
SC
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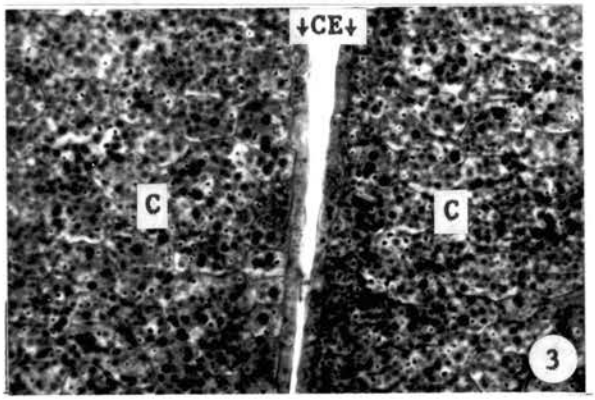
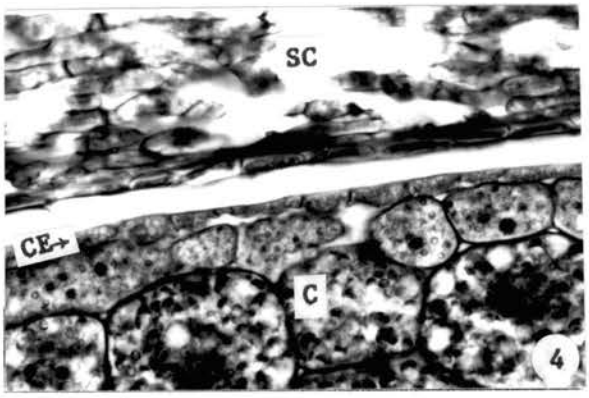
2


↓CE↓
C
C
3


SC
CE
C
4

hybrid embryos and endosperms appeared to be retarded as indicated by their delayed growth. Seeds of the wild x cultivated hybrids grew to maturity while no success was achieved in obtaining mature seeds from the cultivated x wild crosses. The fruits (pods) developed normally but the seeds failed to develop in the latter crosses.

Hyperplastic activity of the maternal, integumentary tissues, particularly the endothelial layer, was observed in aborting ovules (Figure 5). The endosperm had ceased development and the few nuclei in the chalazal region were being encroached upon by the integumentary tissues. The embryo failed to differentiate into the "heart-shaped stage."

Although actual chromosome counts were not made, it is assumed that the chromosome complements of the endosperm differed between the two types of hybrids made in this study, and that the chromosome numbers were identical in the hybrid embryos (30 chromosomes). The chromosome numbers of the endosperms should have been 40 and 50 for the wild x cultivated and the cultivated x wild crosses, respectively. The embryos aborted in the former cross but they appeared normal in the latter one, suggesting that the difference in chromosome number may relate to the cause of embryo abortion. However, insufficient evidence is reported in this study to determine the specific causes of embryo abortion and seed failure. It is suggested, based on this study, that seed failure is associated with retarded embryo and endosperm growth, failure of the embryo to differentiate and hyperplasia of the integuments and eventually the collapse of the endosperm and embryo.

Summary

Development of the embryo and endosperm was observed in ovules

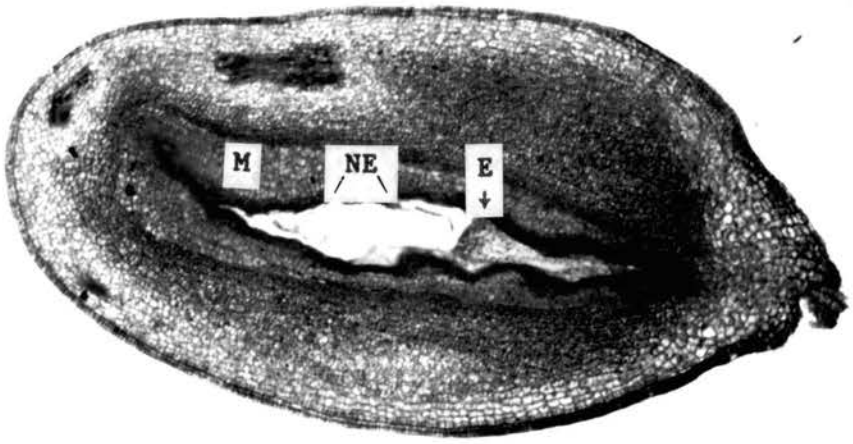


Figure 5. Hyperplastic Activity of Maternal Tissue in an Aborting Ovule. (E = embryo, NE = non-cellular endosperm, M = encroaching maternal tissue).

involving interspecific hybrids and hand pollinated selfs. The endosperm was found to be of the nuclear type, becoming cellular during later stages of its development. Contrary to reports by other investigators the endosperm appeared to be present as a single layer which covers the cotyledons in mature seeds.

Mature seeds were obtained from crosses with A. hypogaea as the pollen parent and the wild diploid A. sp., P-1540, was the female (seed) parent. However, the growth of the embryo and endosperm was observed as being delayed or retarded when compared to the hand pollinated selfs. No success was obtained in crosses where the tetraploid cultivated species, P-112, was the seed parent. Indications of abortions occurred quite early, usually prior to 21 days after pollination.

It was suggested that the differences in the chromosome complements of the respective endosperms in the interspecific crosses may relate to the cause of the embryo abortion. However, this study did not offer enough information to determine the significance of the chromosome number differences between Arachis hypogaea and A. sp. as the cause of embryo abortion.

CHAPTER III

HISTOCHEMICAL ANALYSES OF NON-CELLULAR PEANUT ENDOSPERM

Introduction

The objective of this study was to identify some of the constituents of peanut endosperm using histochemical procedures. The non-cellular endosperm of the peanut is an ideal tissue for such studies because "smears" may be rapidly and easily obtained from this liquid material.

Several histochemical methods were reviewed concerning their specificity to particular chemical components (i.e. starch, protein, amino acids, etc.). Of the methods considered, four were used in this study.

Materials and Methods

Comparable size ovules were collected from ten genotypes: P-112, P-161, P-326, P-935, P-936, P-1284, P-1540, P-1562, P-1563, and P-2395. Ovules were placed on a clean microscope slide, cut in half and the entire volume of liquid endosperm allowed to flow onto it. The endosperm was spread evenly on the slide by using the cut ovule. Several slides were made for each genotype and size. One half of the slides of each were allowed to air dry and the remaining ones immediately killed and fixed with "Spray-cyte" (a water soluble cytological fixative). The smears were stored in a dust free container until they were stained.

The following histochemical methods were used:

(1) Iodine Potassium Iodide (IKI): specific for starch (25).

(2) Periodic acid-Schiff's (PAS) reaction: specific for soluble carbohydrates (25).

(3) Sakaguchi reaction: specific for proteins containing arginine (25).

(4) Triple Stain: specific for deoxyribonucleic acid, polysaccharides, and proteins (22). This procedure involves the use of three separate staining methods (22); Feulgen reaction, PAS, and napthol yellow S.

All histochemical reactions were performed on fixed and air dried smears of each genotype and size.

Results and Discussion

Histochemical methods 1 and 2 gave positive results for starch. However, the latter reaction was superior since the stain was more intense and permanent. A preliminary test involving method 3 (Sakaguchi reaction) failed to produce positive results. However, perfection of this reaction should prove useful since differences between genotypes in regard to the presence of arginine were reported in the amino acid analyses (Chapter VII).

The results using method 4 (Triple Stain) were similar in all slides from each genotype. The nuclei (DNA) stained blue-green; nucleoli (RNA), yellow; protein, yellow-green; and polysaccharides, red. Figure 6 shows the staining reaction on a fixed endosperm smear of P-935. This staining procedure proved to be superior to all of the others because of the clear differentiation of several chemical components.

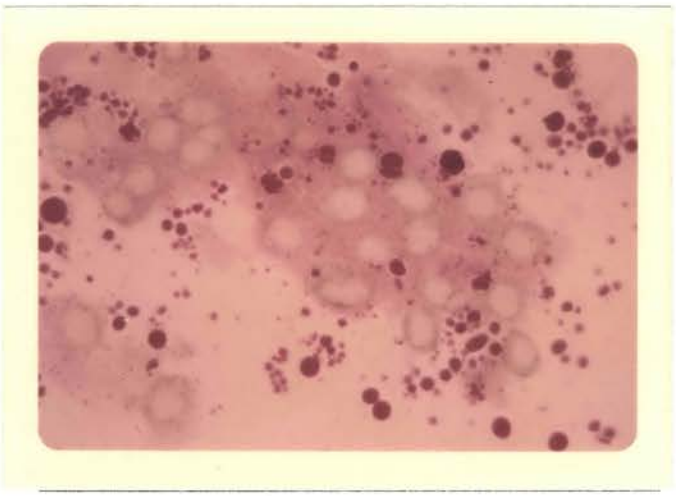


Figure 6. Non-cellular Endosperm of P-935 Showing Staining Reaction of Method 4 (Triple Stain).

Summary

No differences were detected between genotypes by use of the histochemical tests employed. It was evident that starch granules were a conspicuous component of the non-cellular endosperm of the peanut. Of the four histochemical methods used in this study, method 4 (Triple Stain) was superior to the others based on its specificity to several chemical components.

CHAPTER IV

X-RAY DIFFRACTION AS A METHOD FOR DIFFERENTIATING BETWEEN ENDOSPERM STARCHES OF DIFFERENT PEANUT GENOTYPES

Differences in starch granule patterns obtained from x-ray has long been recognized as a method for differentiating between starches of different plant genera (4). Several types of x-ray diffraction patterns have been identified and used to classify starches. Cereal starches yield patterns which have been designated as type "A", potato or tuber starches type "B", and a group of intermediate starches not conforming to either of the two previous classes are designated type "C". It is not known what type is characteristic of starches from the endosperm or cotyledons of the peanut.

Badenhuizen (2) reported that the crystalline structure of starch granules, as revealed by x-ray diffraction patterns, is under genetic control. Brown, Creech, and Johnson (10) also reported genetic control of starch granules and reported differences in x-ray diffraction patterns between different genotypes in maize.

A preliminary study, with a known starch type (maize), utilizing x-rays was made to verify procedures that should give positive results. A commercial corn starch (Argo brand) slurry was spread on a regular microscope slide and x-rayed with a General Electric RX D-6 x-ray diffractometer equipped with a copper target, nickel filter, and 1^{o} beam

collinator, HR Sellar slit, and 0.2° receiving slit. The results were plotted on a strip chart recorder using a time constant of one second and a full scale reading of 200 counts per second. Patterns were recorded through the angular range 1° to 28° 2θ. The results obtained were similar to those of Brown et al.(10)

X-ray diffraction patterns were then attempted on peanut endosperm to detect differences between genotypes and to classify their starch granules as to type. Non-cellular endosperm extracted from ovules of P-112 (cultivated) and P-1540 (wild) were used. Two attempts were made using the entire endosperm (approximately 10 μℓ) from each ovule and the same number of attempts using endosperm from 10 ovules (approximately 100 μℓ) of both wild and cultivated peanuts. However, all attempts to obtain positive x-ray diffraction patterns with peanut starch failed.

It was concluded that the quantities of peanut starch from the endosperm were insufficient to give x-ray diffraction patterns with the previously described x-ray equipment. The use of powder photographs, a more sensitive technique utilizing photographic film and longer x-ray exposure times, might be more effective for these studies (4).

CHAPTER V

FLUOROMETRIC MEASUREMENT OF LIPASE ACTIVITY IN THE NON-CELLULAR ENDOSPERM OF PEANUTS

Introduction

This study was aimed at detecting possible differences in the enzymatic activity of lipid degradation to fatty acids in the endosperm of several genotypes. Lipids are considered to be the esters of fatty acids and related substances such as oil. Although it is well known that peanut cotyledons contain a considerable quantity of oil, (up to 50% or more) (23), the presence of lipids in the endosperm is unknown. Since the endosperm is responsible for the nutrition of the developing embryo, it was suspected that lipids might be detected.

Jacks and Kircher (24) described a rapid and sensitive method to assay lipolytic activity in various oleaginous seeds, including peanuts. They found that fatty acyl esters of 4-methylumbelliferone (7-hydroxy-4 methylcourmarin) are some of the most intensily fluorescent substances known and thus highly sensitive for fluorometric measurement. Guibault, Sadar, and Arcenaux (21) evaluated various fluorometric substrates for lipase activity and suggested 4-methylumbelliferone butyrate (4-MUB) as one of the better substrates for lipase activity. In fact, they found that lipase in concentrations as low as 0.004 mg/ml could be detected with the butyrl ester of 4-MUB.

Pancholy (30) stated that the fluorometric method of lipase

determination was approximately one thousand times more sensitive than the standard fatty acid titration method.

The principle of measuring lipase activity fluorometrically is based on the ability of the enzyme to convert nonfluorescent 4-MUB to highly fluorescent 4-MU.

Materials and Methods

Fresh stock solution (1×10^{-3}M) of 4-MUB was prepared in ethylene glycol monoethyl ether. One hundred microliters of the stock MUB (1×10^{-3}M) was added to one liter of buffered water solution with a pH of 7.41 to obtain the working solution (1×10^{-7}M MUB).

To measure the lipase activity, a Turner III Fluorometer equipped with a 7-60 primary filter and 2A and 47B secondary filters was used. The attenuation dial was set at one and the measurements were recorded continuously at room temperature for 1, 2, and 3 minutes.

Four milliliters of the working solution (1×10^{-7}M 4-MUB buffer) were added to each tube and the fluorometer zeroed before the endosperm (enzyme system) was added. The entire endosperm from one ovule was then added to the buffer in 2 to 10 µl volumes depending on the size ovule to be examined (Table II). The endosperm was added to the buffer by cutting one ovule in half and the liquid, non-cellular, endosperm allowed to flow down the inside of the tube. The starting time was recorded and the endosperm mixed 1-2 seconds with a vortex mixer and immediately placed in the fluorometer.

Endosperm from ovules at three stages of development for seven genotypes were examined for lipolytic activity (Table II). Two samples were run for each size and genotype. The readings (fluorescent units)

were recorded from the instrument dial at 1, 2, and 3 minute intervals. The readings were also recorded graphically by the plotter attachment.

Results and Discussion

The results of this experiment are reported in Table II. Readings followed by an asterisk are considered invalid. Most cases of invalidity were caused by accidently contaminating the sample with portions of peanut cotyledons. Based on the results, some lipolytic activity was detected and differences in activity may offer a means of differentiating between peanut genotypes. However, it appears that insufficient amounts of enzyme were present in the endosperm from single ovules to accurately detect such differences. Further preliminary studies should be made by utilizing endosperm from more than one ovule to validate the results.

The specific location of enzymatic activity might also be observed by preparing fresh endosperm smears on microscope slides, treating them with 4-MUB and observing the activity with fluorescent microscopy. Thus, differences between genotypes might be detected by the location of enzymatic activity.

TABLE II

FLUOROMETERIC MEASUREMENTS OF LIPOLYTIC ACTIVITY

Genotype	Ovule size (cm)	Approx. amount Endosperm (µl)	Fluorescent Readings[a] 1 min.	2 min.	3 min.
P-112	.3 x .15	2	0.5	1.5	2.5
			---	---	---
	.6 x .3	6	4.0	4.5	5.5
			---	---	---
	.8 x .4	10	2.0	4.0	5.5
			15.0	15.0	16.0*
P-161	.4 x .2	5	3.0	3.5	5.0
			5.0	5.0	6.5*
	.6 x .3	6	7.0	7.0	7.5*
			10.0	12.0	15.0
	.8 x .4	10	7.5	8.0	11.0
			8.5	9.0	11.5
P-935	.4 x .2	5	8.5	11.5	15.0
			6.5	7.0	6.0*
	.6 x .3	7	23.5	30.5	37.0
			16.0	15.5	21.5*
	1.0 x .5	8	43.5	47.5	48.5
			7.5	7.0	7.0*
P-936	.4 x .2	5	4.5	5.0	6.0
			7.0	7.5	8.5
	.8 x .3	10	7.5	8.5	10.0
			7.5	9.0	11.0
	1.3 x .5	8	7.5	11.0	14.0
			28.0	41.0	52.0*
P-1284	.4 x .2	5	5.5	6.5	10.0
			11.0	10.0	17.0*
	.6 x .3	7	11.5	8.5	14.0*
			18.0	20.0	29.0

Table II Continued

Genotype	Ovule size (cm)	Approx. amount Endosperm (µℓ)	Fluorescent Readings[a] 1 min.	2 min.	3 min.
	1.0 x .4	8	12.5	25.0	34.5
			15.0	11.0	20.5*
P-1286	.3 x .2	2	5.5	6.5	8.0
			8.5	10.0	8.0*
	.4 x .3	5	10.5	13.5	15.0
			17.5	21.0	24.0
	.7 x .5	7	8.5	10.5	12.0
			5.0	7.0	7.5
P-2395	.3 x .15	2	7.5	7.0	9.5*
			5.0	4.5	8.0*
	.9 x .3	10	8.0	7.0	8.0*
			38.0	37.0	54.0*
	1.2 x .6	8	30.5	27.0	41.0*
			22.0	24.0	38.0

[a]--- = no sample

* = invalid reading (see text)

CHAPTER VI

DETERMINATION OF STARCH GRANULE SIZE DISTRIBUTION PATTERNS FROM NON-CELLULAR PEANUT ENDOSPERM

Introduction

Studies of starch granule size distribution patterns are useful in identifying starches from different plant genera. For example, corn and potato starches are easily identified and can be differentiated from each other by their size distribution patterns (36). However, insufficient information is available concerning differences in starch granule size distribution patterns at the species and sub-species level of most plant genera.

Preliminary observations of peanut endosperm revealed that the starch granules are abundant constituents and are variable in size, ranging from less than 1 μ to about 8 or 9 μ. The only other conspicuous particles in the endosperm, disregarding the cytoplasmic strands, were the nuclei which were 10 μ to about 20 μ in size.

The question arose as to whether or not differences in peanut genotypes could be detected by measuring the sizes of the starch granules both at the species and sub-species level. Endosperm from six cultivated genotypes, collected at three stages of development (approximately 14, 21 and 28 days old) and one wild species collected at two stages of development (approximately 14 and 28 days old), were examined

for the frequencies of sizes of their starch granules.

One method of cell or particle counting is by microscopic observation using a haemocytometer, a special slide often used for counting blood cells. This slide allows estimates to be made of cell numbers but it does not differentiate according to sizes. Use of a haemocytometer is both time consuming and subject to substantial error (10% or more).

Materials and Methods

For this study a Model B Coulter Counter was used rather than a haemocytometer because it is faster and subject to less error. The particle size determinations obtained with the Coulter Counter are based on the volume of the particles regardless of their shape. The instrument is able to count up to 100,000 particles per sample (½ ml) in 13 seconds.

The Coulter Counter was calibrated with latex particles 3.49 μ in diameter using a 100 μ aperture. The procedure for calibration and instrumentation was in accordance with the manufacturer (15). For the peanut starch granule studies the following instrument settings were used:

Gain.....50
Amplification.....1/8
Aperture current.....1/4
Vernier.....100
Lower threshold.....17.1
Mode switch.....separate

The instrument was manually adjusted by setting the threshold dials to achieve the desired classes of particle sizes (0.94, 1.80, 3.10, 3.90, 5.20, 6.00, 6.90, and 8.20 μ).

Two plants of each genotype were harvested and used in this study.

The ovules were removed from the pods of the plants and the basal ovules grouped in appropriate classes according to three stages of development: Age 1 (young), Age 2 (intermediate), Age 3 (old). These ages correspond closely to 14, 21, and 28 day old ovules.

The endosperm was extracted from the ovules by using a "Zeptrol" pipette which proved to be superior to other pipettes and syringes that were tried. Fifty microliters of endosperm were removed from several fresh ovules (five to twenty depending upon size) from each of the three age groups. The endosperms were mixed with equal volumes of absolute ethanol to inhibit organism growth that might affect the starch properties. However, use of the ethanol may not have been necessary owing to the fact that counts were made within 12 hours after the endosperm was prepared.

Immediately prior to making the counts, the 100 μℓ of endosperm solution (endosperm + ethanol) from each genotype and age group were mixed with 20 ml of an electrolyte (NaCl) and placed in a clear plastic vial. The vial was placed on the counter stand and the aperture tube immersed in it. The instrument automatically used a ½ ml aliquot of the total solution (endosperm + ethanol + electrolyte) for each count. Two counts for each threshold setting were made for each genotype and age.

Results and Discussion

The results are graphically reported in Figures 7, 8, 9, and 10 for Age 1 (young), Age 2 (intermediate) Age 3 (old), and total counts, respectively.

Figure 7 shows the results obtained for the Age 1 ovules of the

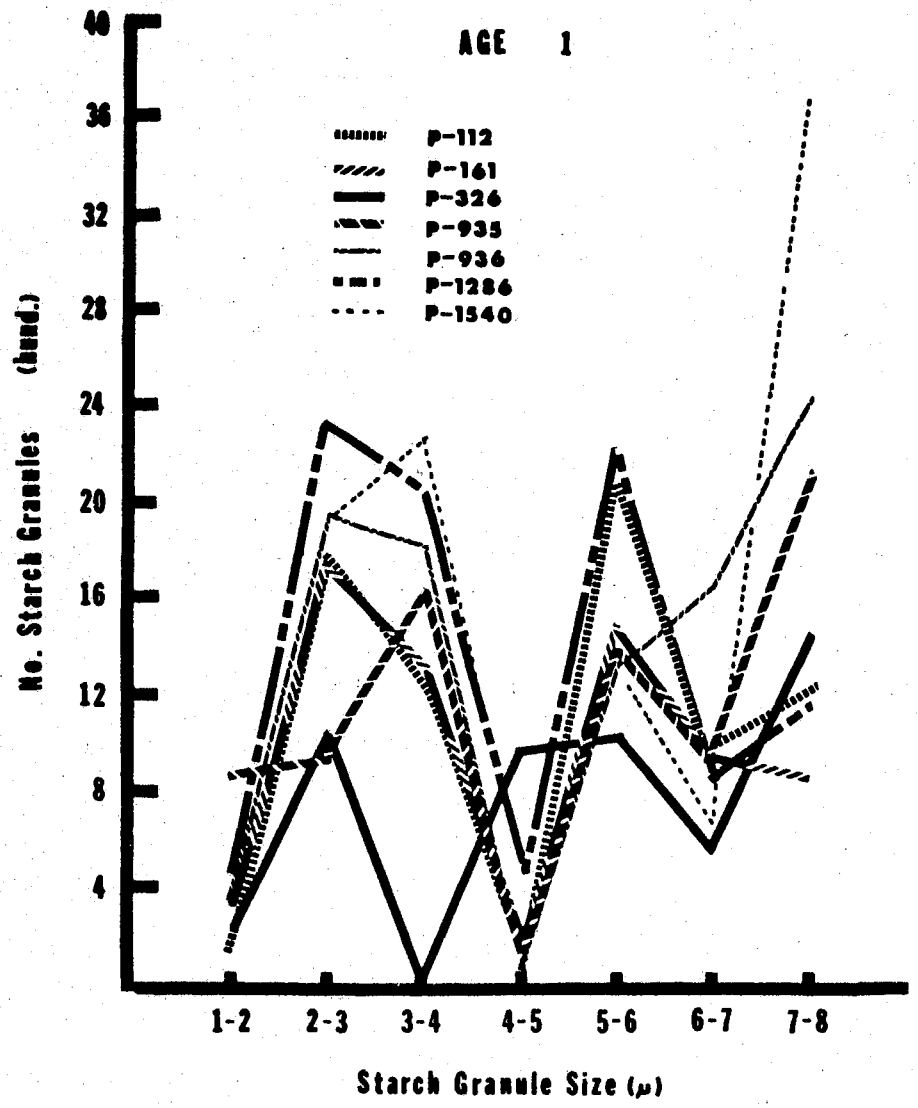


Figure 7. Size Distribution Patterns of Starch Granules From Endosperm of Seven Genotypes at Age 1.

seven genotypes. The general pattern of starch granule size frequencies of most genotypes are similar with differences shown only in the number of starch granules. The only striking differences in this respect appears in the P-326, where no starch granules were present in the 3-4 μ range. The low count in the other genotypes occurred in the 4-5μ class. Another obvious difference occurred in the 7-8 μ size class where P-1540 had considerably more starch granules than the other genotypes studied. The other genotypes showed similar trends with only slight differences in the number of starch granules at each class.

Figure 8 shows the results of the Age 2 (intermediate) ovules. The wild species, P-1540, was omitted from this study because no intermediate size ovules were collected. The same general patterns occurred here as in the previous study. Note, however, that P-326 did not differ from the other genotypes at the 3-4μ size class as it did in the previous figure. However, P-161 was different from the other genotypes at the 3-4 μ size class. Comparisons can be made regarding the numbers of granules present at each size range for each genotype. The greatest contrast between genotypes appeared in the granule size range of 6-7μ and 7-8 μ. Note that in the former range P-326 had more than 10 times the number of starch granules than P-936; but in the latter range a reverse trend occurred. Similar trends can be detected for P-112 and P-1286 at the same two previously mentioned size ranges.

Figure 9 shows the results for the Age 3 ovules. All genotypes again show the same general trends. However, it appears that there are slightly greater differences between the genotypes in this age group than in the younger ovules. Again, all genotypes followed the same trend until the 5-6, 6-7, and 7-8 μ size classes, where the greatest

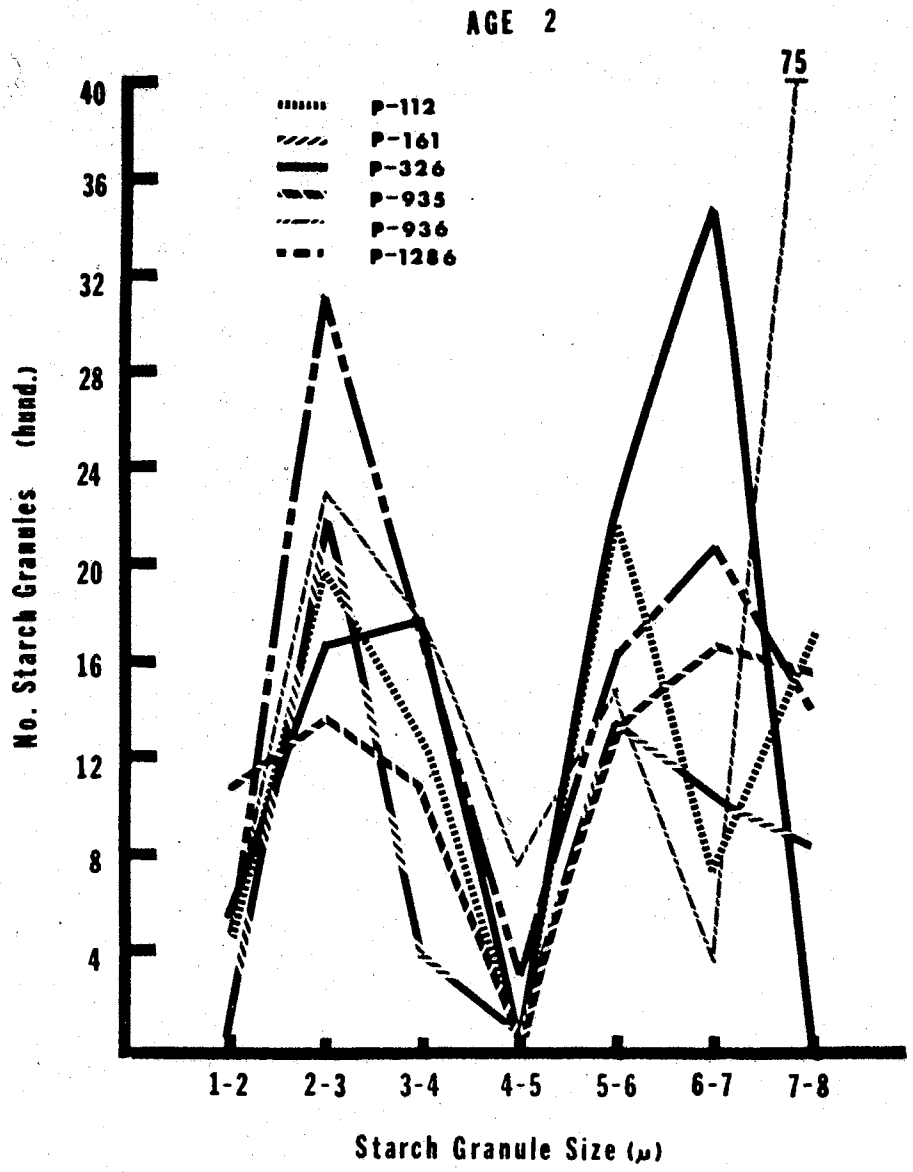


Figure 8. Size Distribution Patterns of Starch Granules From Endosperm of Six Genotypes at Age 2.

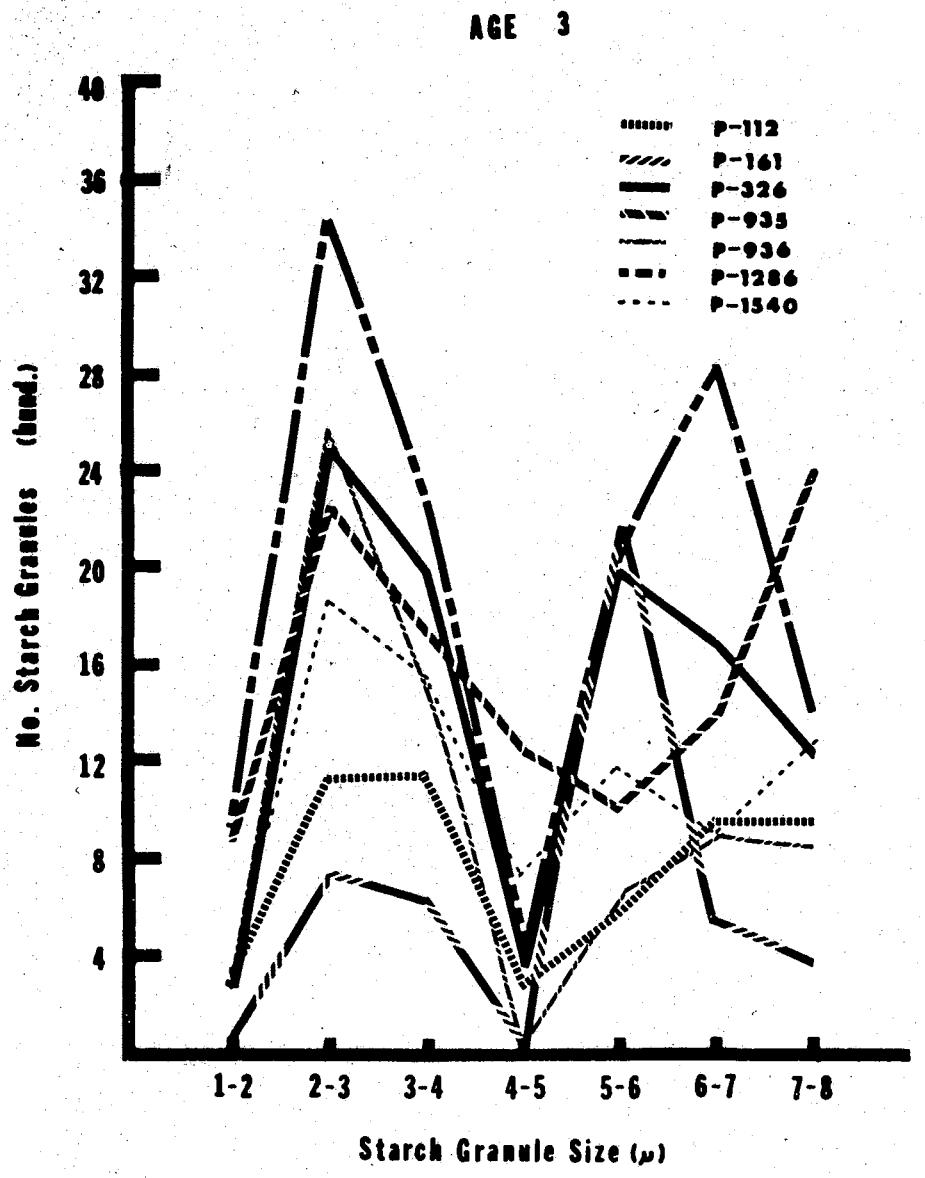


Figure 9. Size Distribution Patterns of Starch Granules From Endosperm of Seven Genotypes at Age 3.

contrasts occurred. Note that certain genotypes showed increases in starch granule numbers at these classes whereas others showed decreases.

Figure 10 shows the total number of starch granules for each genotype according to age. This figure summarizes the previous three graphs with respect to age and genotype. It omits, however, the differences (previously observed) in particle size. Again, numerous comparisons can be made; for example, compare the total starch granules at Age 1 for each genotype and note that P-1540 had the greatest number and P-326 had the least. Similar comparisons can be made for each age and genotype. Note the trends in the number of granules with respect to age within a particular genotype. For example, in P-112 and P-936 as the ovules matured, the total number of granules increased and then decreased. The total number of starch granules decreased as P-161 ovules became older but the opposite was true for P-326, P-935, and P-1286.

If the total number of starch granules reported in Figure 10 is converted to numbers per milliliter, taking the dilution factor into consideration, they would range from 3.7 million to 12.2 million. Thus, the abundance of these food reserves is apparent. Figure 10 may offer some significant information regarding the production of starch granules. The differences shown here by genotypes may have a bearing on the ability of the endosperm of a particular genotype to nourish its embryo. It is known that crosses between some parents fail more often than do crosses between other parents (both intraspecifically and interspecifically). The significance of the low granule numbers in the 4-5 μ range in all ages that were observed (except P-326 at Age 1) is not known. It appears that production or growth of the starch granules may be cyclic in nature.

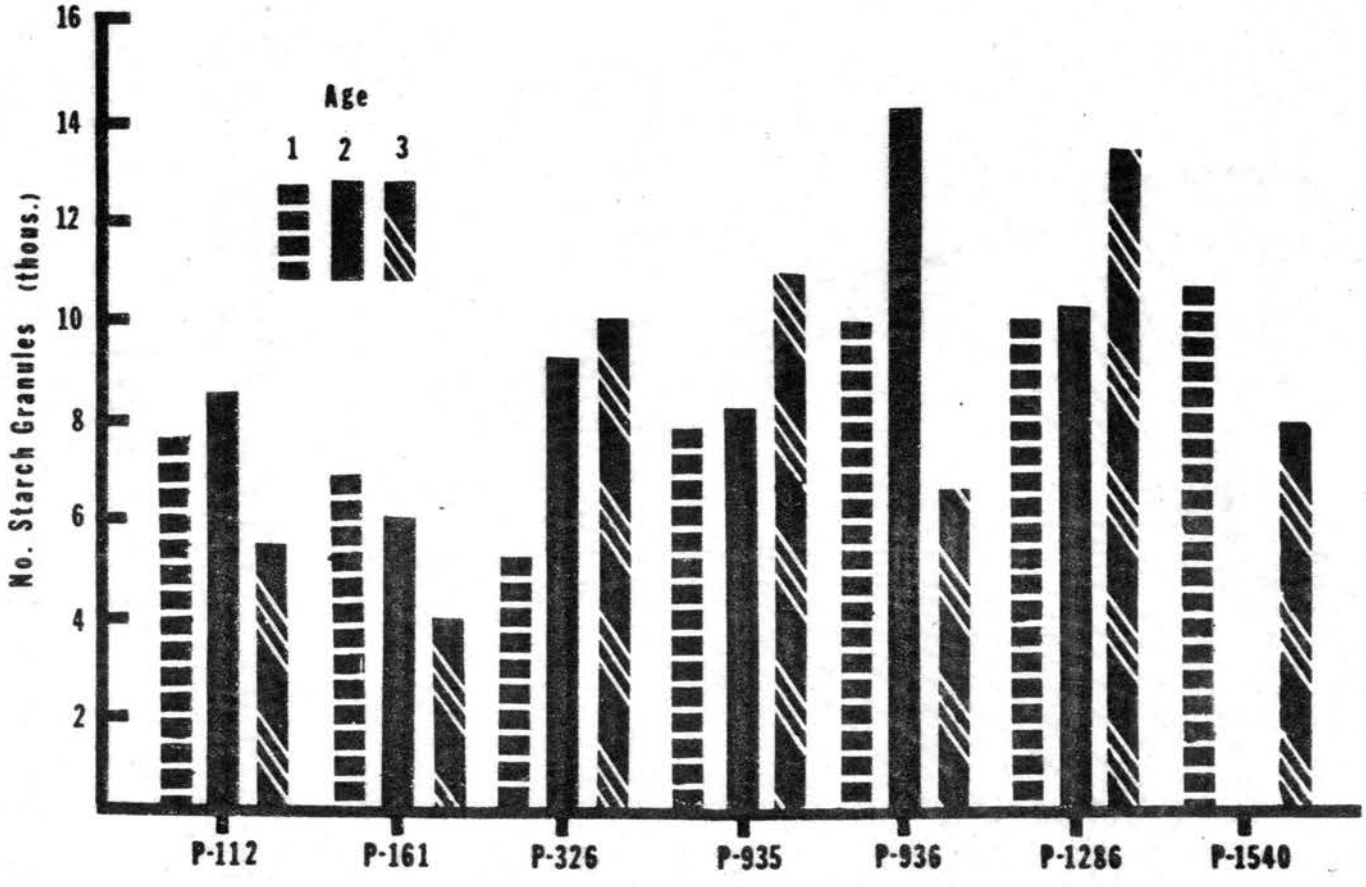


Figure 10. Total Number of Starch Granules for Each Genotype According to Age.

It is not known what factors caused the differences that were observed within the particular age group in regard to particle size, or in total starch granules. However, from these data, it appears that some differences do occur between genotypes in regard to starch granule sizes in their endosperms. These differences may be controlled genetically.

The exact ages of the ovules examined were unknown because the flowers were not tagged at the time of pollination. Therefore, the ovules from the various genotypes may have differed, somewhat, in physiological maturity. These differences in age could account for some of the results that were obtained.

CHAPTER VII

BIOCHEMICAL DIFFERENCES IN ENDOSPERMS FROM SEVERAL PEANUT GENOTYPES

Introduction

It is known that chemical differences between genotypes exist because gene expression in phenotypes occur by way of biochemical pathways in organisms (35). Biochemical differences have been reported at the species level (19, 35, 43), but few references have been made to varietal differences (35).

Fredriksson (19) recorded biochemical differences between species of Festuca based on the phenolic compounds that he separated with cellulose thin-layer chromatography. Towers and Maass (43) found differences in phenolic acids of Lycopodiales at both the species and subspecies level. Differences in flavonoid compounds from peas and beans have been reported by Rowlands and Corner (35). A biochemical approach, employing thin-layer chromatography to separate and detect differences in fluorescent compounds in Avena, was characterized by Grant and Whetter (20). Bell (6) reported chemical differences in Lathryus after examining 49 species for ninhydrin reacting compounds. Duvick (17) examined four varieties of maize for free amino acids at various ages of development to detect differences in these nitrogenous compounds at the sub-species level.

For this study, several experiments were employed in order to

detect chemical differences in the endosperm of peanuts at both the species and sub-species level.

Experiment 1

Preliminary thin-layer chromatograms for carboxylic acid, amino acids, reducing sugars, estrogens, steroids, and phenolics were prepared on the endosperms of two cultivated (P-112 and P-161) and one wild (P-1540) genotypes to determine if chemical differences could be detected.

Materials and Methods

Pods were harvested in August from plants grown in pots in a greenhouse, with a temperature variation of 21 to 29 C. Ovules were removed from the pods, washed and grouped into two sizes (0.6 x 0.3 cm and 0.3 x 0.2 cm). Ovules of the two groups from each genotype were cut in half in 400 µl of 95% ethanol in a watch glass. The fluid was quickly pipetted into ½ dram vials. Additional 95% ethanol was added to the vials to bring the total liquid volume up to 1.0 ml, and the vials were placed in a freezer at 10 C until used.

Within 24 hours, 75 µl of each endosperm extract was spotted on precoated silica gel 20 x 20 cm glass plates (Brinkman Silplate-22), dried, and put in chromatography tanks containing solvents for the specific tests (39). Three plates with endosperm from each genotype and size were prepared in one dimension for each test.

Results and Discussion

Results of the preliminary chromatograms are reported in Table III. Carboxylic acid, estrogens, steroids and phenolics were not detected in

TABLE III

RESULTS OF PRELIMINARY THIN-LAYER CHROMATOGRAMS FOR DETECTION OF CHEMICAL DIFFERENCES IN PEANUT ENDOSPERM

Genotype	Ovule Size (cm)	Approx. Amount Endosperm (µℓ)	Carboxylic Acid	Amino Acids	Reducing Sugars	Estrogens	Steroids	Phenolics
P-112	0.6 x 0.3	25	(-)	(+)	(+)	(-)	(-)	(-)
	0.3 x 0.2	10	(-)	(+)	(+)	(-)	(-)	(-)
P-161	0.6 x 0.3	25	(-)	(+)	(+)	(-)	(-)	(-)
	0.3 x 0.2	10	(-)	(+)	(+)	(-)	(-)	(-)
P-1540	0.6 x 0.3	5	(-)	(+)	(-)	(-)	(-)	(-)
	0.3 x 0.2	5	(-)	(+)	(-)	(-)	(-)	(-)

(+) = detected

(-) = not detected

any of the genotypes that were examined. Reducing sugars were faintly detectable in both cultivated genotypes. However, even though not indicated in the table, reducing sugars may have been present in P-1540 but the concentrations may have been too small to detect. All the genotypes examined showed intense color reactions for amino acids; but no attempt was made to identify them.

Because free amino acids were detected, further analyses, including use of an amino acid analyzer (AAA) and thin-layer chromatography (TLC) were made. (Experiments 2, and 3-4, respectively).

Experiment 2

Materials and Methods

Two cultivated genotypes (P-112 and P-326) and one wild genotype (P-1540) were analyzed for free amino acids by using a Beckman Model 120C Amino Acid Analyzer. Ovules of P-112 and P-326 were cut in half and 30 µl of non-cellular endosperm extracted with a "Zeptrol" pipette and mixed with 2.0 ml of 70% ethanol in a centrifuge tube. The solutions were centrifuged for three minutes and the supernate pipetted into 10.0 ml beakers and vacuum dried (10 inches of mercury) at room temperature. The same procedure was followed for P-1540, except that only half as much endosperm and ethanol was used owing to the small quantity of P-1540 ovules.

The dried P-112 and P-326 samples were then mixed with 2.0 ml of lithium citrate buffer (pH 2.1) and 1.0 ml of this mixture was analyzed for free amino acids. Only 1.3 ml of the lithium citrate buffer was added to the dried P-1540 sample. However, the same quantity as above (1.0 ml) was used for the analysis. Thus, since the endosperm samples

of the wild and cultivated peanuts were of the same dilutions and the same quantity was analyzed, their results would be directly comparable.

Results and Discussion

Quantitative and qualitative results from the amino acids analyzer are reported in Table IV and Figure 11. P-112 and P-326 were fairly similar in regard to the kinds of amino acids present in their endosperms. However, some slight differences occurred in the amounts (μ-moles) of amino acids present in these two genotypes. For instance, P-112 contained about three times as much asparagine and about ten times as much isoleucine as P-326. P-1540 showed some striking differences from the cultivated genotypes in amino acids both quantitatively and qualitatively (Figure 11). Compare P-1540 with P-112 and P-326. Note that valine, methionine, and arginine have large peaks in the two latter genotypes, but these amino acids are absent in the former one. P-1540 crosses successfully when it is used as a female parent in interspecific crosses with A. hypogaea. However, when P-1540 is used as the pollen parent it will not hybridize with A. hypogaea (See Chapter I). Perhaps the absence of valine, methionine, and arginine in the endosperm of P-1540 is related to its ability to cross as a female with A. hypogaea. Further investigations in this area are warranted. The reason other amino acids were not detected in P-1540 may be due to inadequate sample size.

Several unknown amino acids were detected in all genotypes. Although the identities of these amino acids are uncertain, it is known (because of the standards that were used in calibrating the instrument) that these peaks are not: citrulline, half cystine, α-aminoadipic

TABLE IV

QUANTITATIVE[a] AND QUALITATIVE RESULTS OF FREE AMINO ACIDS IN NON-CELLULAR PEANUT ENDOSPERM AS DETECTED BY AN AMINO ACID ANALYZER

Amino acid	Genotypes P-112	P-326	P-1540
Asp	0.0210	0.0201	(*)
Thr	0.0730	0.0747	(*)
Ser	0.1306	0.0747	0.0050
Asn	1.5130	0.5701	0.3570
Glu	(*)	(*)	(*)
Gln	0.7210	0.7183	0.0167
Pro	0.2146	0.1826	(*)
Gly	0.0348	0.0319	(*)
Ala	0.3089	0.4742	0.0361
α-H_2N But	0.0020	(-)	(-)
Val	0.1413	0.1146	(-)
Met	0.0335	0.1117	(-)
Ile	0.3415	0.0350	0.0059
Leu	0.0144	0.0188	(*)
Phe	0.0266	0.0081	(*)
His	0.0136	0.0113	(-)
Arg	0.0558	0.0845	(-)

[a] reported in μmoles

(-) = amino acid absent

(*) = amino acid present in small amount or not measurable

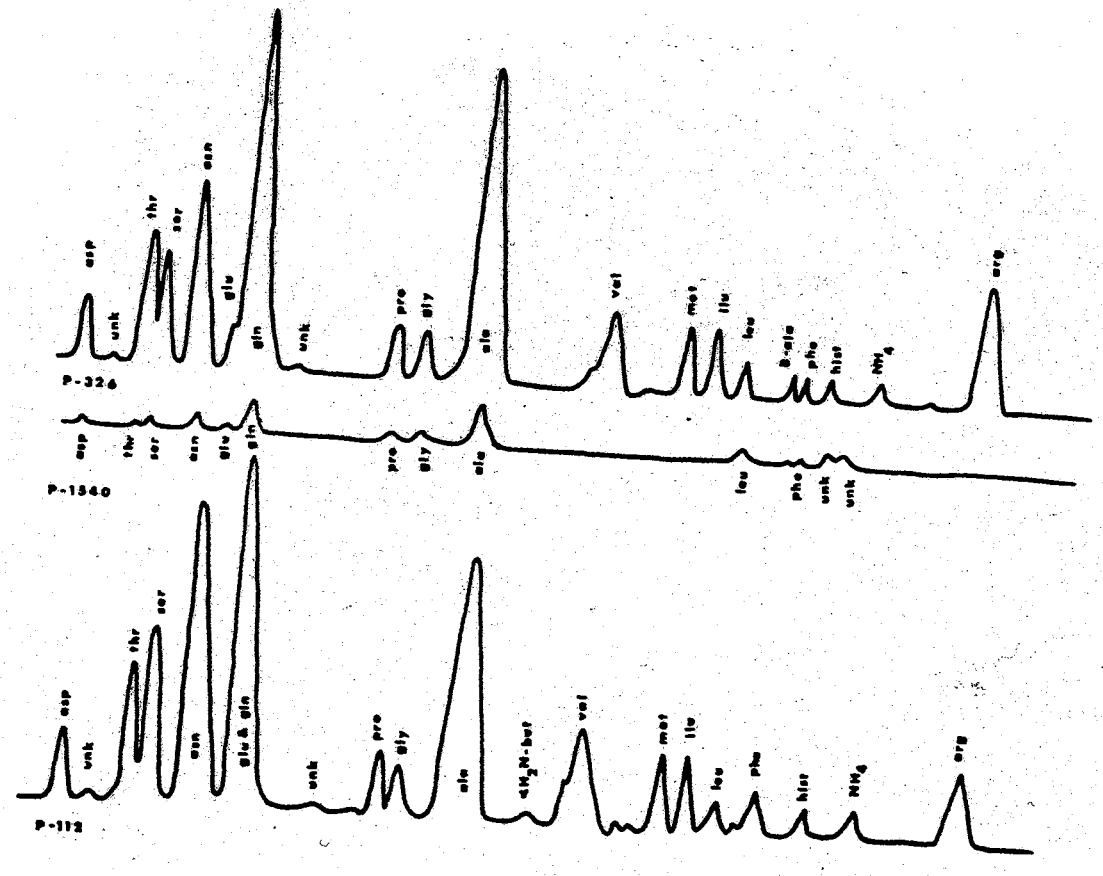


Figure 11. Free Amino Acids Detected From Non-Cellular Peanut Endosperm.

acid, α-amino-normal-butyric acid, phosphoserine, hydroxyproline, ornithine, cystathionine, or γ-aminobutyric acid.

From these results it appears that biochemical differences in free amino acids in peanut endosperm occur at the species level. Differences may also occur at the sub-species level although these are not quite as apparent.

It is interesting to compare the free amino acids detected in peanut endosperm in this experiment with the amino acids that were detected in maize endosperm and peanut cotyledons by Duvick (17) and Young (45), respectively. Free amino acids detected in the endosperms of peanuts and maize and the cotyledons of peanuts were: glutamic acid, aspartic acid, alanine, leucine, isoleucine, serine, valine, proline, glycine, asparagine, glutamine, histidine, and arginine. Threonine was reported in both maize and peanut endosperm but not in peanut cotyledons. Methionine, phenylalanine and ammonia were detected in peanut endosperm and cotyledons but not in maize endosperm. Lysine, cystine, methylene glutamic acid, and methylene glutamine were detected only in peanut cotyledons.

Thus, it appears that the endosperms in some plant species from different genera are quite similar in free amino acid composition. More differences were apparent in amino acid content between peanut endosperm and peanut cotyledons than between the endosperms of maize and peanuts. As mentioned above, several amino acids were reported in peanut cotyledons by Young (45) that were not detected in peanut endosperm in the present study. These differences may be due to the function of the two tissues. The cotyledons are constantly building and storing proteins while they are utilizing the amino acids in the endosperm.

Experiment 3

Further determinations of free amino acids were conducted using thin-layer chromatography while looking specifically for the absence of valine, methionine, and arginine in some additional genotypes at both the species and sub-species level.

Materials and Methods

The analyses made on endosperm extracts from the wild and cultivated genotypes are presented in Table V. Ovules were removed from the pods of plants from each genotype and grouped according to size. Endosperm was extracted by a microliter syringe which was inserted into the chalazal region of the ovules. (No attempt was made to collect the same amount of endosperm from each genotype because the thin-layer chromatogram is highly sensitive quanitative measure). The extracted liquid, non-cellular endosperms were placed in ½ dram vials, mixed with 1.0 ml of 70% ethanol and kept in a freezer at 10 C until they were used.

The procedure used in this experiment was developed by Bujard and Mauron (11). The ethanol-endosperm extract (50 µℓ) was spotted on precoated 20 x 20 cm cellulose glass plates (Brinkman Celplate 22), 2.0 µℓ at a time using a "Zeptrol" pipette. Valine, methionine, and arginine were used as control amino acids prepared in the following manner: 0.003 grams of each amino acid was dissolved in 3.0 ml of 70% ethanol. Twenty five microliters of each amino acid were spotted on a control cellulose plate in the same manner as described for the endosperm extracts.

After the plates were spotted, they were placed in chromatography tanks previously saturated with the solvent vapors. The solvent systems

TABLE V

DIVERSE GENOTYPES USED FOR TLC DETECTION OF FREE AMINO ACIDS

Genotype	Ovule Size (cm)	Approx. Amount Endosperm (µl)
P-112	0.6 x 0.3	50
P-161	0.6 x 0.3	35
P-326	0.7 x 0.3	45
P-935	0.6 x 0.3	100
P-936	0.8 x 0.3	35
P-1284	0.6 x 0.3	35
P-1286	0.4 x 0.3	35
P-2395	0.7 x 0.3	25
P-1562	0.7 x 0.2	30
P-1563	0.5 x 0.2	30
P-1540	0.7 x 0.3	30

used were:

FIRST DIMENSION---Methanol-Chloroform-17% Ammonium hydroxide (2:2:1)

SECOND DIMENSION--Methanol-Water-Pyridine (20:5:1)

The plates were removed after the solvent had reached a height of 15-17 cm (approximately 240 minutes) and allowed to air dry at room temperature. The plates were placed in the second dimensional solvent until the solvent had reached the edge of the plates (approximately 100 minutes). The plates were then removed from the tanks and allowed to air dry at room temperature. After they had dried, they were sprayed with 0.1% ninhydrin and heated in an oven at 100 C for 15 minutes to develop the amino acid spots. Many of the amino acids produced specific colors which permitted easy identification. The amino acids were identified according to the procedure of Bujard et al. (11). The spots were outlined in ink for easier differentiation of the amino acids.

Results and Discussion

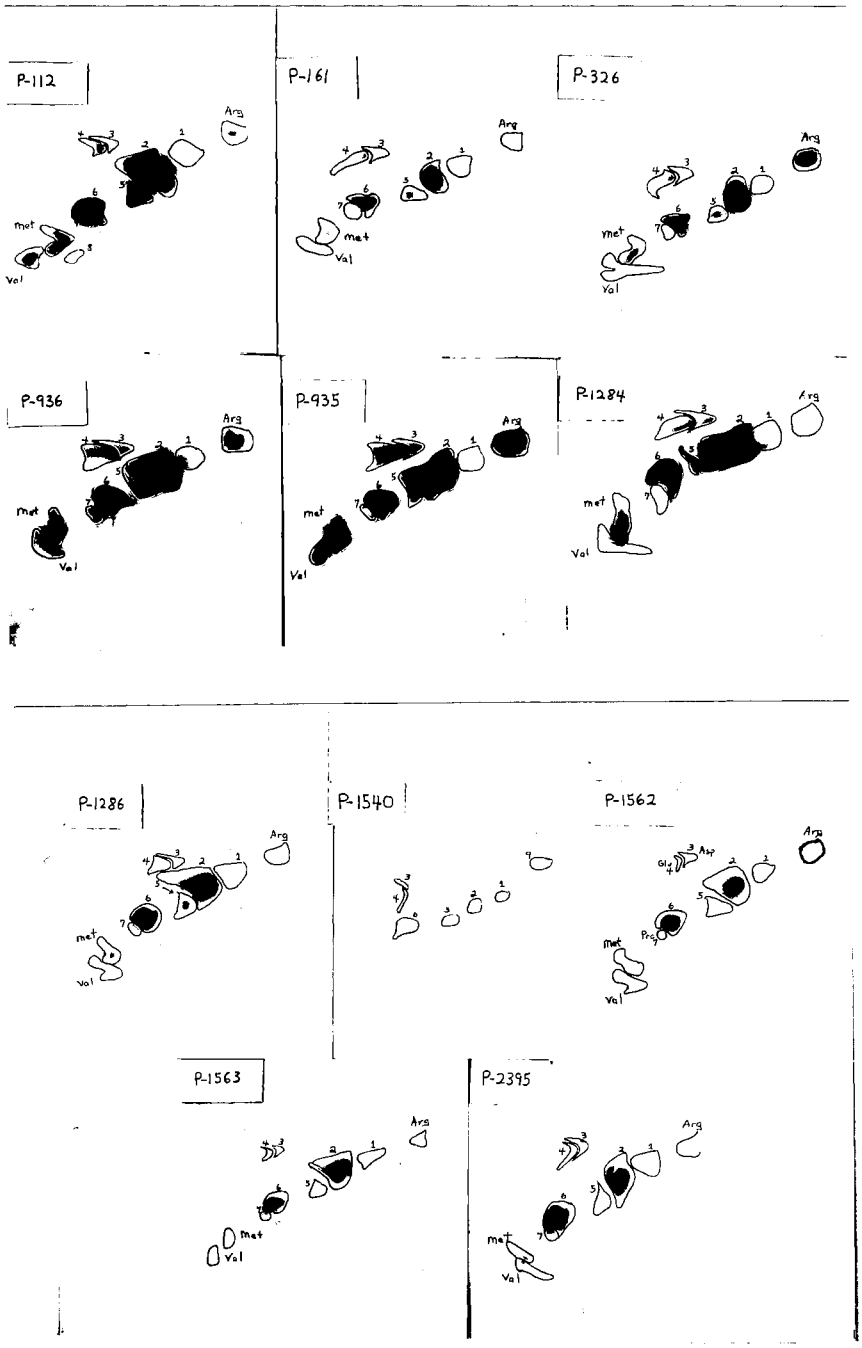
The amino acids that were detected in the peanut endosperms of the various genotypes examined are reported in Table VI. Figures 12a and 12b are photographs of the chromatograms showing the amino acid separations for the eleven divergent genotypes. The results of the chromatograms confirmed that valine, methionine, and arginine were absent in the endosperm of P-1540 as had been indicated by the AAA. P-1540 was the only genotype which showed a spot thought to be homoarginine. Homoarginine is a naturally occurring guanidino amino acid which is present in the seeds of many legumes (6). The AAA did not analyze for this non-protein amino acid because the samples were inadvertently removed from the analyzer before this material was due to be detected. Additional

TABLE VI

FREE AMINO ACIDS FROM NON-CELLULAR PEANUT ENDOSPERM AS DETECTED BY TLC

Amino acid[a]	Genotypes										
				Cultivated					Wild		
	P-112	P-161	P-326	P-935	P-936	P-1284	P-1286	P-2395	P-1540	P-1562	P-1563
Arginine	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
Methionine	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
Valine	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
2	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
3	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
4	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
5	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
6	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
7	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
8	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)
9	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)

[a]
1 = histidine
2 = serine, glycine, and glutamine
3 = aspartic acid
4 = glutamic acid
5 = alanine and threonine
6 = tryptophan
7 = proline
8 = leucine and isoleucine
9 = homoarginine



Figures 12a & 12 b. Photographs of Chromatograms Showing the Separations of Free Amino Acids of Eleven Genotypes.

samples should be tested to positively establish the absence or presence of homoarginine in various peanut endosperms.

Based on the results of the thin-layer chromatograms, P-112 was the only genotype with endosperm containing leucine and isoleucine. However, the results from the AAA showed the presence of these two amino acids in P-326. Of the two methods used (TLC and AAA) the amino acid analyzer is probably more reliable because of its greater sensitivity to amino acids (concentration below 0.002 μmoles). Therefore, it is suggested that leucine and isoleucine may be present in the other genotypes.

Experiment 4

The previous thin-layer chromatograms were obtained by spotting an aliquot of endosperm from several ovules (four to twelve depending on size) of each genotype. A more useful method would be to use endosperm from single ovules for each chromatogram so that the production of various amino acids could be followed in sequence throughout the development of the endosperm. This experiment was conducted to determine if a single ovule might be used to obtain chromatograms of the amino acids present in peanut endosperm.

Materials and Methods

The endosperm contents (7 μℓ) from one ovule (0.6 x 0.3 cm) of P-112, that had been mixed with 21 μℓ of 70% ethanol, was spotted on a cellulose plate and processed as previously described. Prior to spraying with ninhydrin, the plate was examined in a UV "Chromato-Vue" (fluorescent chamber) to detect fluorescent spots.

Results and Discussion

The chromatogram (Figure 13) shows the presence of all amino acids that had been previously identified by TLC methods for P-112. In fact, the plate was slightly overloaded (i.e. excess amounts of amino acids resulted in the spots running together). The wavy circles shown in Figure 13 indicate fluorescent spots which were noted when the plate was exposed to UV light prior to spraying with ninhydrin. The identity of these spots is not known and no attempts were made to identify them for this study. However, these spots may offer another approach to detecting biochemical differences. From this test it appears that single ovules do produce sufficient amounts of amino acids in their endosperms to be detected by TLC. Employment of this technique may be invaluable for determining the importance of certain amino acids in the nutritional aspects of embryo development.

Summary

Preliminary TLC studies produced evidence that free amino acids are abundant in the non-cellular endosperm of peanuts. Experiments involving identification of the amino acids with an amino acid analyzer revealed the absence of valine, methionine, and arginine in the endosperm of one wild species (P-1540). Single ovules were found to contain sufficient endosperm to allow for amino acid separation and identification on thin-layer cellulose plates.

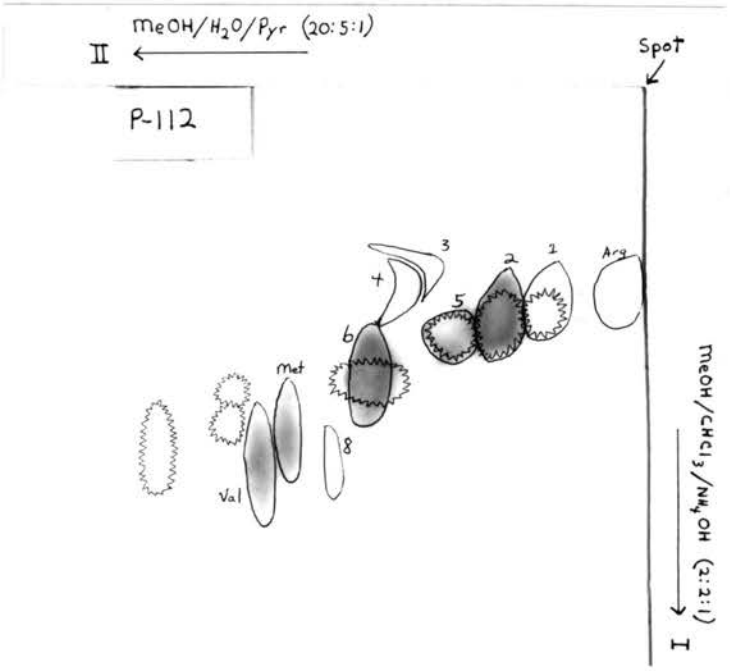


Figure 13. Photograph of a Chromatogram of Free Amino Acid Separation From Endosperm of One Peanut Ovule.

CHAPTER VIII

A COMPARATIVE STUDY OF THE BIREFRINGENCE END-POINT TEMPERATURES OF THE NON-CELLULAR ENDOSPERM FROM SEVERAL GENOTYPES OF PEANUTS (ARACHIS HYPOGAEA L.)

Introduction

It was reported in Chapter III that starch granules are conspicuous constituents of peanut endosperm. Subsequently, x-ray diffraction, Chapter IV, and starch distribution studies, Chapter VI, and the following experiment were initiated to determine whether or not genetically diverse peanut genotypes might possess different kinds of starch granules.

Badenhuizen (2) reported that in plant taxa, the shape, average size, strength, and crystalline patterns of starch granules are influenced by different genes. Other investigators have also reported genetic control of starch properties in plants (10, 28, 31). Brown, Creech, and Johnson (10), after examining some mutants of maize, presented evidence based on x-ray diffraction and birefringence end-point temperature (BEPT) data, that showed that the physical structure of the starch granules in the developing endosperms was under genetic control.

Several methods have been described for ascertaining BEPTs (16, 18, 36). All of these, however, rely on the same principle; when a suspension of starch granules is heated, the granules swell, burst, and

lose their anisotropy or birefringence (called the gelatinization point by some authors). This phenomenon is easily detected by examining the starch granules microscopically under polarized light as they are heated and observing the disappearance of the "Nichols cross" (10). The technique used in this study was similar to that described by Schoch and Maywald (36).

Materials and Methods

Eight genotypes of peanuts, chosen because of their distinctive plant, fruit, and seed phenotypes, were used in this study. They were: P-112, P-161, P-326, P-935, P-936, P-1284, P-1286, and P-2395. Several pods from two plants of each genotype, collected at various stages of maturity, were taken to the laboratory and washed in tap water. The basal ovules were removed from the pods and grouped by size into three classes, (1) young (0.3 x 0.15 cm), (2) intermediate (0.6 x 0.3 cm), and (3) old (0.8 x 0.4 cm). These sizes correspond fairly closely, according to other studies, to 14, 21, and 28 day old ovules (after pollination). Endosperm starch from each ovule was obtained by cutting the fresh ovule in half with a razor blade on a clean microscope slide and allowing the liquid endosperm to flow onto the surface. The approximate amounts of endosperm used were 2, 6, and 10 µl for the young, intermediate, and old ages, respectively. The starch granules were not isolated from the endosperm, but were used in their natural fluids (except for the salt treatment below). A salt treatment was applied to one group of the age 3 ovules by adding one drop (approximately 30 µl) of 1 M $Ca(NO_3)_2$ to the endosperm on the slide. This salt treatment was used because Pfahler et al. (31) had shown that the starches from some maize

genotypes could be further differentiated by this procedure. The endosperm on the slide was ringed with a high viscosity mineral oil and a cover glass was placed on it such that the starch suspension was completely surrounded by the oil without the presence of air bubbles. The slide was immediately (except in the case of the salt treatment where observations were delayed for five minutes) placed on a microscope equipped with a Kofler hot stage and polarizing filters. The rate of temperature increase of the hot stage was about 2 C per minute (transformer set at 23 volts). Each of the microscopic fields (x312) contained several hundred starch granules. The temperature was recorded when the first three to four granules lost their birefringence (initial BEPT). The final BEPT was recorded when all but two or three granules showed this character. Two samples--each sample consisting of one ovule--were examined for two plants of each genotype for each age and treatment (salt vs. no salt).

Results and Discussion

The results are presented graphically in Figures 14 and 15. Figure 14 shows the initial BEPT of the starches from the various peanut genotypes by age and treatment. At age 1, P-161 had the lowest BEPT and P-1286 had the highest. Note that the BEPT of all genotypes was lower for age 2 than for age 1 except for P-161 and P-1286 where the reverse was true or P-935 where essentially no change occurred. At age 3, P-112 alone, showed a distinct increase in BEPT while the other genotypes either decreased or remained essentially the same. P-326 showed a decrease in BEPT as the endosperm matured but P-935 showed about the same BEPT at all ages.

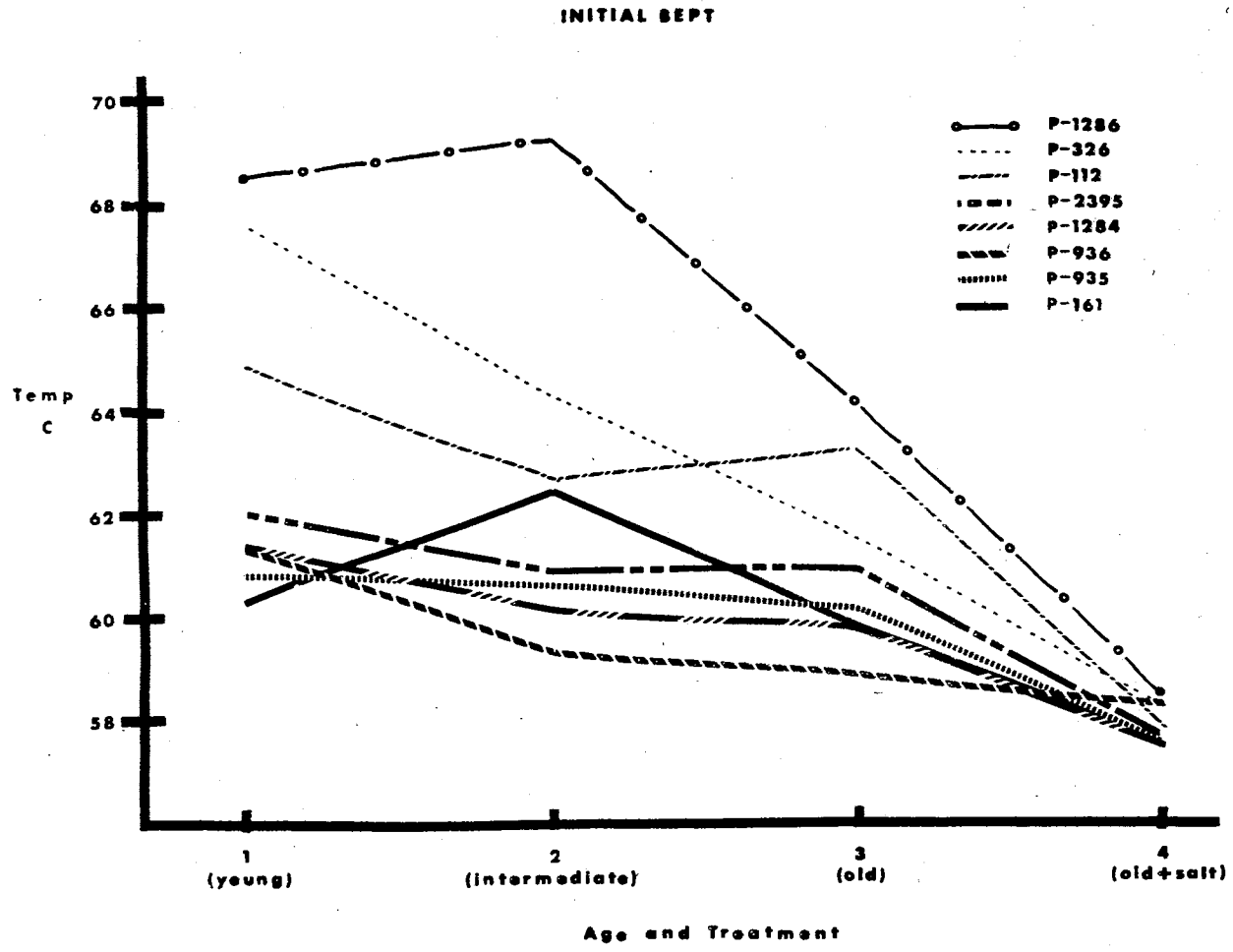


Figure 14. Initial Birefringence End-point Temperature (BEPT) of Starch Granules From Ovules of Eight Genotypes by Age and Treatment.

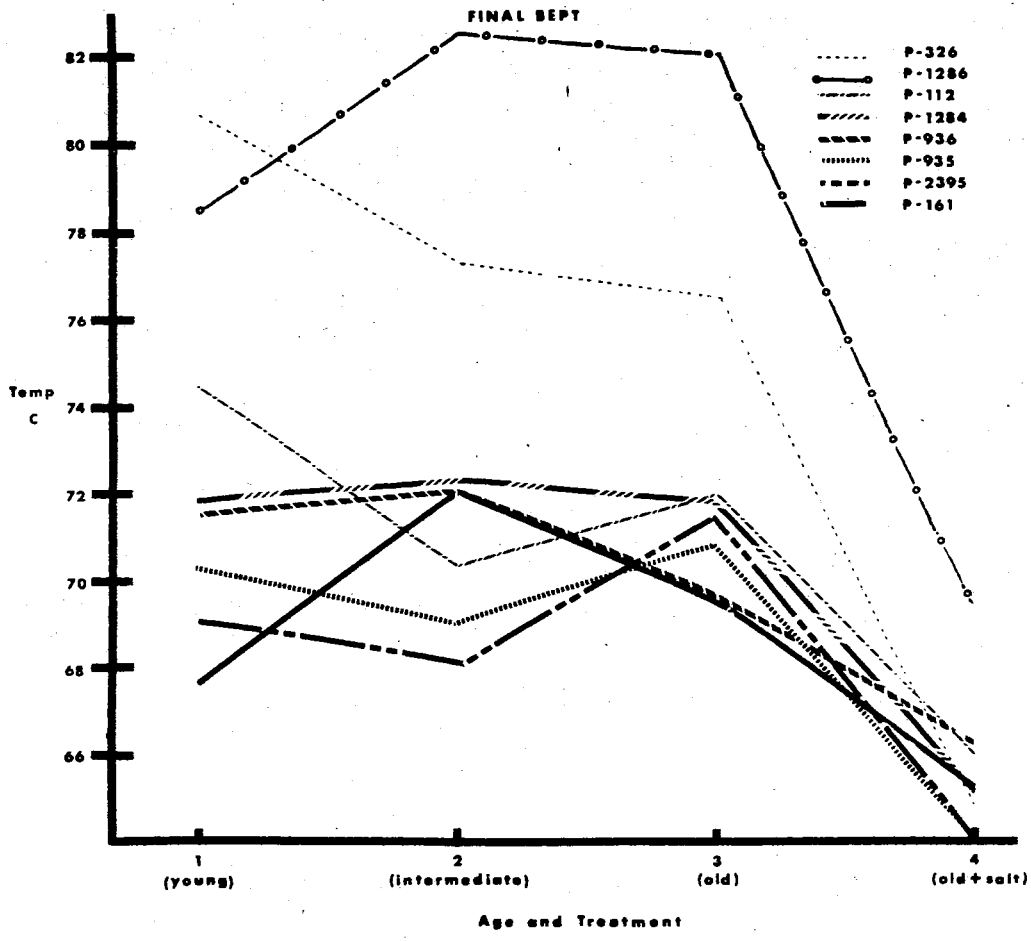


Figure 15. Final Birefringence End-point Temperature (BEPT) of Starch Granules From Ovules of Eight Genotypes by Age and Treatment.

Figure 15 shows the final BEPTs. P-112, P-326, P-935, and P-2395 showed a decrease in BEPT from the young to the intermediate age. The other genotypes showed increases in BEPT at these ages. P-326 and P-1286 showed significantly higher final BEPTs for all ages (without salt) than did the other genotypes. The latter had final BEPTs below 75 C. Note the contrast of P-161 with P-112 BEPTs as their endosperms of different ages were tested. The former showed an increasing then decreasing BEPT with age, while in the latter the reverse trend was apparent. P-935 and P-2395 reacted similarily to P-112 but at slightly lower temperatures.

The salt treatment depressed both initial and final BEPTs of all genotypes except for P-936 where little effect was noted in the initial BEPT.

Two methods, Duncan's Multiple Range Test and the Neuman-Keul Test (42) were employed to determine whether or not the results obtained were statistically significant from each other. The latter test is easier and faster to compute than the former and it tends to be more conservative. Tables VII, VIII, and IX show the results of the statistical analyses for initial, final, and the final minus initial BEPTs, respectively. Many comparisons are possible with these data, however, some significant conclusions are as follows:

1. The starch granules of P-326 and P-1286, although they are not distinct from each other (except for initial BEPT at age 2, based on Duncan's Test), are obviously different from several of the other genotypes examined with respect to both their initial and final BEPTs at ages 1 and 2. It is interesting that these two are the most distinct genotypes used in this study in regard to their overall plant

TABLE VII

MEAN INITIAL BIREFRINGENCE END-POINT TEMPERATURES (BEPT) OF PEANUT ENDOSPERM STARCH FROM EIGHT GENOTYPES AT THREE AGES AND ONE SALT TREATMENT

Genotype	Initial BEPT[a] Endosperm age[b] and treatment[c] 1	2	3	4
P-112	64.75 QR pqrs	62.63 RSTUV pqrstu	63.25 RSTU pqrstu	57.88 YZ stu
P-161	60.38 UVWXYZ stu	62.50 RSTUV pqrstu	59.88 UVWXYZ stu	57.63 Z u
P-326	67.63 PQ pqr	64.63 QRS pqrst	61.50 RSTUVWX rstu	58.38 XYZ stu
P-935	60.75 TUVWXYZ rstu	60.63 UVWXYZ stu	60.13 UVWXYZ stu	57.50 Z u
P-936	61.25 TUVWXY rstu	59.38 VWXYZ stu	58.75 WXYZ stu	59.25 VWXYZ stu
P-1284	61.38 STUVWX rstu	60.13 UVWXYZ stu	59.75 VWXYZ stu	57.75 Z tu
P-1286	68.50 P pq	69.25 P p	64.13 RST pqrstu	58.50 XYZ stu
P-2395	62.00 RSTUVW qrstu	60.88 TUVWXYZ rstu	60.88 TUVWXYZ rstu	57.63 Z u

[a] In centigrade. Means not followed by a common letter are significantly different at .05 according to the Duncan Multiple Range Test (capital letters) and the Neuman-Keul Test (small letters).

[b] 1 = young, 2 = intermediate, 3 = old (approximately 14, 21, and 28 days after pollination, respectively).

[c] 4 = age 3 + 1 M $Ca(NO_3)_2$.

TABLE VIII

MEAN FINAL BIREFRINGENCE END-POINT TEMPERATURES (BEPT) OF PEANUT ENDOSPERM STARCH FROM EIGHT GENOTYPES AT THREE AGES AND ONE SALT TREATMENT

	Final BEPT[a]			
	Endosperm age[b] and treatment[c]			
Genotype	1	2	3	4
P-112	74.50 QRSTU pqrstu	70.38 STUVW pqrstu	72.00 RSTUV pqrstu	66.00 VW stu
P-161	67.63 UVW stu	72.00 RSTUV pqrstu	69.50 TUVW pqrst	65.38 VW tu
P-326	80.75 PQ pqr	77.38 PQRS pqrst	76.50 PQRST pqrst	64.88 VW tu
P-935	70.38 STUVW pqrstu	69.00 UVW qrstu	70.88 STUVW pqrstu	64.00 W u
P-936	71.50 RSTUV pqrstu	72.00 RSTUV pqrstu	69.63 TUVW pqrstu	66.63 VW stu
P-1284	71.88 RSTUV pqrstu	72.25 RSTUV pqrstu	71.88 RSTUV pqrstu	65.00 VW tu
P-1286	78.50 PQR pqrs	82.63 P p	82.13 P pq	69.50 TUVW pqr stu
P-2395	69.13 TUVW qrstu	68.13 UVW rstu	71.75 RSTUV pqrstu	64.00 W u

[a] In centigrade. Means not followed by a common letter are significantly different at .05 according to the Duncan Multiple Range Test (capital letters) and the Neuman-Keul Test (small letters).

[b] 1 = young, 2 = intermediate, 3 = old (approximately 14, 21, and 28 days after pollination, respectively).

[c] 4 = age 3 + 1 M $Ca(NO_3)_2$.

TABLE IX

MEAN FINAL MINUS INITIAL BIREFRINGENCE END-POINT TEMPERATURES (BEPT) OF PEANUT ENDOSPERM STARCH FROM EIGHT GENOTYPES AT THREE AGES AND ONE SALT TREATMENT

Genotype	Final minus Initial BEPT[a] Endosperm age[b] and treatment[c] 1	2	3	4
P-112	QRS 9.75 pq	RS 7.75 pq	QRS 8.75 pq	RS 8.13 pq
P-161	RS 7.25 pq	QRS 9.50 pq	QRS 9.63 pq	RS 7.75 pq
P-326	PQR 13.13 pq	PQRS 12.75 pq	PQ 15.00 pq	S 6.50 pq
P-935	QRS 9.63 pq	RS 8.38 pq	QRS 10.75 pq	S 6.50 pq
P-936	QRS 10.25 pq	PQRS 12.63 pq	QRS 10.88 pq	RS 7.38 pq
P-1284	QRS 10.50 pq	PQRS 12.13 pq	PQRS 11.13 pq	RS 7.25 pq
P-1286	QRS 10.00 p	PQR 13.38 pq	Q 18.00 pq	QRS 11.00 pq
P-2395	RS 7.13 pq	RS 7.25 q	QRS 10.88 pq	S 6.38 pq

[a] In centigrade. Means not followed by a common letter are significantly different at .05 according to the Duncan Multiple Range Test (capital letters) and the Neuman-Keul Test (small letters).

[b] 1 = young, 2 = intermediate, 3 = old (approximately 14, 21, and 28 days after pollination, respectively).

[c] 4 = age 3 + 1 M $Ca(NO_3)_2$.

phenotypes. (See Table I).

2. The greatest differentiation of genotypes by initial and final BEPTs occurred at ages 1 and 2, according to both statistical tests.

3. Final minus initial BEPTs were ineffective in distinguishing between genotypes within ages.

Brown et al. (10) found the greatest differentiation of maize genotypes by starch granule BEPTs occurred at the older ages (24 days after pollination). However, in the tests reported here differences were found in peanuts at the earlier ages. These variances may be due to the differences in the physiological changes that take place in the starch granules of these two diverse taxa. Starch in maize endosperm is accumulated as a food material to be used later by the germinating and developing seedling; whereas in peanuts, the endosperm is of short duration and is essentially absent in the mature seed. Hence, a more logical comparison might be starch from maize endosperm versus starch from peanut cotyledons.

The significance that starch granule structure may have on biological behavior or adaptation in various plant species is unknown. However, it does appear that some peanut genotypes differ with respect to their starch granule structure and these differences are probably under genetic control. A study of hybrids made between some of the genotypes tested might help elucidate the genetics involved.

Summary

Initial and final birefringence end-point temperatures (BEPTs) were determined for the starch granules of the non-cellular endosperm from ovules at three stages of development from diverse peanut

genotypes. The results indicated that P-326 and P-1286 possess starch granules that are different from the other genotypes studied. The greatest differentiation was noted at ages 1 and 2 (approximately 14 and 21 days after pollination) for both initial and final BEPTs. The differences that were detected are assumed to be genetically controlled.

CHAPTER IX

SUMMARY

A better understanding of the factors controlling normal endosperm development and the possible detection of genetically controlled differences between endosperms of several diverse peanut genotypes were the main objectives of this study. Two sections were devoted to these objectives:

Section 1 Endosperm Morphology

Anatomical observations were made on ovaries, pegs, and ovules of interspecific hybrids (made reciprocally) and hand pollinated selfs in an effort to study embryo and endosperm development. The endosperm was found to be present as a single layer of cells between the seed coat and cotyledons of mature peanut seeds. These findings contradict previous reports (12, 32). Hybrid endosperms and embryos of crosses between A. hypogaea (P-112) and A. sp. (P-1540) were found to be retarded when compared to the endosperms and embryos from developing selfs. Hyperplastic activity of the endothelium was observed in aborting ovules of the cultivated x wild crosses where the former was the female parent. The exact causes of embryo abortion and resultant seed failure were not detected from these studies.

Section 2 Endosperm Composition

Several studies were conducted in an effort to identify various

components of the peanut endosperm and possibly to detect genetically controlled differences between endosperm of several divergent genotypes. Starch was found to be a conspicuous component of the endosperm. P-326 and P-1286, as revealed by BEPT, appeared to have starch granules which differed greatly from the other genotypes examined. Variations in starch granule size by genotype were observed in a size distribution study of endosperm starches at several stages of development. The x-ray diffraction studies of endosperm starch and the fluorometric measurements of lipolytic activity in the endosperm were unsuccessful techniques for detecting differences between endosperms of several peanut genotypes. Biochemical differences between two species of Arachis were detected by amino acid analyses. Non-cellular endosperm of P-1540 was found to be devoid of valine, methionine, and arginine. These results suggest that differences in the chemical composition of developing endosperms may be of considerable importance in the reproductive behavior of various peanut taxa.

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VITA 2

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