VOLUMETRIC CHANGES AND THEIR IMPLICATIONS FOR TISSUE COLLAPSE IN A LEAFSPOT OF AN *ARACHIS* INTERSPECIFIC HYBRID

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PREFACE

A stereological point counting morphometric analysis was performed on cross sections of peanut leaves which showed chlorotic spotting. Chlorenchymatous tissues degraded earlier than nonchlorenchymatous tissues. This was associated with a translucent appearance which was due to loss of pigments.

Many people assisted me with my research endeavors. I wish to express my deepest appreciation to my major adviser, Dr. Paul E. Richardson, for his help and support throughout this project. At times, his assistance was 'above and beyond the call of duty'.

I would also like to thank the members of my committee, Dr. Becky Johnson and Dr. Hassan Melouk, for the time they have contributed toward this study. Other help was provided by Dr. Ali Al-Mousawi for my training on the ultramicrotome. Financial assistance from the National Science Foundation's Undergraduate Research Program during preliminary studies is appreciated.

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CHAPTER I

INTRODUCTION

Interspecific hybridization between cultivated crops and wild species from geographical centers of origin furnishes a means of incorporating desirable genomic characters which make the plant better adapted to man's needs (16). There are a number of wild peanut taxa in South America, some of which exibit either tolerance or resistance to pests, *e.g.*, mites (17,18), insects (3,19,32), viruses (5,15) and fungi (4,14,34).

One species, *Arachis chacoense* Krap *et* Greg. *nom. nud.*, has shown promising resistance to *Cercospora arachidicola* Hori (1,11,12,13,20,21,28). However, frequent leaflet degeneration occurs in alloploid plants when hybridized with *A. hypogeae* L., the cultivated peanut (29). Leaves of these hybrids exibit chlorotic lesions and tissue collapse is externally apparent.

The goal of this investigation was to document microscopic changes associated with tissue collapse, and to relate this to the development of chlorotic lesions. An experiment was conducted to determine what volumetric tissue changes occurred in diseased as opposed to healthy leaflet areas from a three-way cross exibiting pronounced lesions,

i.e., *A. hypogeae* cv. Chico, *A. chacoense* (PI 276235), and *A. stenosperma* Krap *et* Greg. *nom. nud.* (PI 338280). Point counting morphometry was used to determine volumetric fractions.

CHAPTER II

LITERATURE REVIEW

Peanut Breeding Research

Some peanut breeding efforts have concentrated on an attempt to produce viable hybrids which would help to clarify the taxonomic and paleobotanical relationships between *Arachis* species. For example, Smartt (29), Smartt and Gregory (30) and Smartt *et al.* (31) crossed wild and cultivated species. These authors have attempted to relate compatibility with evolutionarily distinguishable taxonomic groupings.

Other researchers have concentrated on incorporating agronomically desirable characteristics into cultivated peanut as means to improve production. Hemmingway (14) screened a number of *A. hypogeae* entries for resistance to *C. arachidicola* and drew conclusions on the structural basis of resistance. Their main conclusion was that resistance decreases with increases in stomatal size. Abdou *et al.* (1) examined a number of species, including *A. chacoense*, and found some selections immune and others highly resistant to *C. arachidicola* and *Cercosporidium personatum* (Beck & Curtis) Deighton. They also related resistance to leaflet

structure and the orientation of the fungal germ tube growth.

Hassan and Buete (13) found that fewer lesions developed when *A. chacoense* genomic material was present in an interspecific cross with another wild species, *A. cardenasii* (greenhouse and field studies). Melouk and Banks (20) found that no lesions appeared six weeks after inoculation with *C. arachidicola* and little leaflet defoliation occurred. Similarly, Sharief *et al.* (28) noted increased resistance to *C. arachidicola* when *A. chacoense* was crossed with *A. hypogeae* or *A. cardenasii*.

Foster *et al.* (11) performed field studies on cultivated and wild peanut species and found that *A. chacoense* had the fewest lesions per leaflet, fewest lesions per 100 square cm leaf area, and least necrotic tissue per unit of leaf area when inoculated with *C. arachidicola*. When Gobina *et al.* (12) inoculated with spore suspensions of *C. arachidicola*, *A. chacoense* and its hybrids developed a few lesions and necrotic spots, but no conidia were recovered. Field studies supported their findings (21).

Smartt (29) observed that ten of twenty-six hybrids of *A. chacoense* and *A. hypogeae* showed leaflet necrosis, of variable severity. Since grafts to normal plants did not transmit the condition, Smartt (29) concluded that the disorder was not viral, but due to "some cytoplasmic particle" transmitted through the pollen, which resulted in the "physiological imbalance causing necrosis."

Banks (personal communication) also observed a high frequency of auto-degeneration in crosses involving *A. chacoense*. We observed that a translucent, chlorotic spotting of leaves, cataphylls, and to a lesser degree, stems was prominent, but varied with age, and possibly with envirnomental conditions. No mention of this phenomenon was made in publications of Hassan and Buete (13), Melouk and Banks (20), Sharief *et al.* (28), Gobina *et al.* (12), Melouk *et al.* (21), or Bharathi *et al.* (2). Bharathi *et al.* (2) described the hybrids of *A. chacoense* and *A. hypogeae* as being "healthy and vigorous", however, photographic plates included in that paper show symptoms similar to those observed in the hybrids tested during this study.

Peanut Leaf Anatomy

The leaf of *Arachis* spp. is pinnately compound with four leaflets. Reed (26) described the normal leaf anatomy of *A. hypogeae*. Leaflet cross-sectional anatomy consists of an upper and a lower epidermis, two to four layers of loosely arranged palisade mesophyll, a few layers of spongy mesophyll, vascular bundles surrounded by bundle sheath with sheath extensions continuous with both upper and lower epidermis, conical cells with tanniferous substances interspersed in the upper epidermis, and a layer of large water storage cells below the spongy mesophyll. Stomata occur with equal frequency on the adaxial and abaxial leaflet surfaces (23). Other authors have described leaf

anatomy of various species, not including *A. chacoense*, but, in general, these descriptions conform to Reed's (*e.g.*, 6,8,23).

Preliminary studies (8,9,10) showed mesophyll collapse in affected areas. Vascular tissue, bundle sheath and extensions, water storage cells, and upper and lower epidermis maintained cellular integrity until late necrotic stages. Plasmolemma and tonoplast damage of mesophyll cells was apparent in electron micrographs. Chloroplast lamellar arrangements were abnormal. Mitochondrial and nuclear membrane breakdown occurred during final stages of cell collapse.

The possibility of peanut mottle virus (PMV) infection leading to these symptoms was tested by a bean local-lesion bioassay (27) in the preliminary studies cited above. All tests showed negative results. Plants used in the current study may have been exposed to PMV during an insect infestation which occurred at the time tissue was fixed, and later studies have shown widespread occurence of PMV in the germplasm collection (22). Thus, symptomology could reflect partial expression of viral, as well as genetic components. However, leaflet symptoms appeared the same as in previous years' experiments.

Other interspecific hybrids have shown genetically induced lethal spotting necroses. Phillips and Reid (25) found leaf lesions, necrotic cells, and mitochondrial degeneration in *Gossypium* spp. hybrids. Phenolic compounds

were associated with cell necrosis and vascular occlusion in the affected tissues of these hybrids.

A morphometric study was undertaken to further analyze the volumetric changes of specific cell types associated with leaf tissue collapse. Comparisons included volume proportions within each disease state, and changes between the healthy and chlorotic leaflet areas. Also, information was related to previous histochemical studies.

CHAPTER III

MATERIALS AND METHODS

In September of 1982, 8 cuttings (15-18 cm long) were taken from field grown plants and gently washed under running tap water. The shoots were supported by foam plugs and rooted in test tubes in half-strength Hoagland solution. These were placed in a plastic covered chamber under a greenhouse bench (30±4 C, 100% RH). After rooting (about 3 weeks), they were washed and planted in 15.4 cm clay pots in a sandy loam soil. They were grown in a greenhouse under natural lighting for 4 months.

In January 1983, tissue samples were cut from the first fully expanded leaf (third from the apex). All 8 plants had developed lesions, but only 5 of the 8 plants had lesions expressed in the chosen leaflets. As is standard practice in our lab, a hypodermic needle with one square mm bore was used to excise tissue samples in .1 M potassium phosphate-buffered 4% gluteraldehyde at 4 C. The samples were fixed in gluteraldehyde for 2 hr, washed, then postfixed with 2% osmium tetroxide for 4 hr. After dehydration in a graded series of ethanol, the tissue was embedded in a firm-formulation of Spurr epoxy resin (33). Eight samples were taken from each of the 5 plants- 4 from healthy and 4 from

chlorotic areas. Healthy samples were taken from an unaffected portion of the same leaflet that contained the chlorotic sample.

Thick cross sections, <0.5 μm, were cut with a Sorvall MT-2 ultramicrotome with glass knives. Two sections with no common cells were taken from each block, stained in 1% Toluidine Blue O in 1% sodium borate (7), then covered and stored for analysis.

Slides were observed on an AO Microstar with an orthoilluminator, and photomicrographs (200X) were taken with Ektachrome 160 tungsten slide film. The area to be photographed was arbitrarily predetermined (center of the section) to decrease bias. In addition, the thickness at the center of the section was measured using a calibrated eyepiece micrometer at 200x. Slides (35 mm) were projected on a Sawyer "Mirascreen" with a slide projector.

A psuedorandom point grid was used to measure the volumetric parameters of the tissue (24). The grid point spacing was determined by the formula: '$a > d^2$' where 'a' = area of component in question, 'd' = distance between points or corners of grid spacing (35). The average palisade parenchyma cell area was used for 'a' to decrease the number of point densities needed for measurements. Since most leaf cells are only slightly larger or smaller than the palisade cells, one grid spacing should decrease the amount of work needed without significantly increasing statistical error. Once the grid spacing was determined (1.5 cm), a table of

random numbers (34) was used to determine coordinates to plot a random point in each of the grid squares. On a transparency, this array of points was randomly placed on the projected image of the section, and point counts of each tissue component were recorded on a multiple hand-tally device.

The length of leaflet section measured was the same whether healthy or chlorotic tissue was viewed. The only tissue change should be in the height due to vertical tissue collapse. Thus the total number of points on each chlorotic component was divided into the total point count on the corresponding healthy cell type to give the fractional volume collapse. For each disease state, the total point count of each cell type was also divided into the total point count of all sections to give its volume fraction.

CHAPTER IV

RESULTS

As seen in Table I, appendix, the number of grid points falling on profiles of each component was less in chlorotic tissues, except for the lower epidermis and vascular tissue. As expected, the number of points on collapsed cells (*e.g.*, chlorechyma and water storage cells) was higher. The substantial change in point counts of chlorenchymatous cells (mainly palisade and spongy parenchyma) can be related to loss of photosynthetic capability in lesioned areas. This was seen under fluorescent microscopy as loss of red chloroplast color (9) and, macroscopically, as chlorosis.

Table II, appendix, lists the volume fractions of the tissue components within each disease state. For example, 8.7% of the healthy tissues was upper epidermis compared to 12.8% in the chlorotic samples. Of interest is the volume fractions of chlorophyllous and achlorophyllous cells. Chlorotic cell types occupied half of the volume occupied in healthy tissues, and the epidermal and vascular tissues are greater in proportion in the chlorotic state fractions as compared to the healthy state. Area occupied by tannin cells, with or without tanniferous contents, decreased.

Table III, appendix, compares the volumetric collapse of leaflet tissues as a whole. For example, leaf tissues showing the chlorotic state had 69% less palisade parenchyma than healthy tissues (100-31). Overall, leaf cross-sectional area in chlorotic lesions occupied about 61% of the volume of that in healthy leaflet areas. This corresponds well to the ratio of the occular micrometer width measurements from the center of the sections. Of interest is the apparent increase in the volume of the lower epidermis. This anomaly will be considered further in the discussion.

Tannin cells were observed in nearly all of the healthy sections, even though the point count method resulted in their not being counted in every section. However, in chlorotic samples, there was a decided lack of cells resembling tannin cells. This is shown in table 3 where there is a 90% decrease in apparent volume. Some of this decrease could be accounted for in the collapsed chlorenchyma category, since collapsed cells in the upper mesophyll layers were hard to distinguish as to their origin. The significance of this will be further discussed.

In abaxial tissue layers, especially in water storage cells, healthy sections showed some collapse, but not to the extent found in chlorotic tissues. This might have been related to the method of tissue sampling, or to some physiological imbalance. Since the method of tissue

selection was on the basis of chlorotic versus green appearance, some cell degredation might have occurred in the apparently healthy tissues. The breakdown of nonchlorenchymatous cells would not have been visually apparent at the time of tissue selection.

CHAPTER V

DISCUSSION

Morphometry is a practical and accurate method for determining volumetric changes associated with many environmental parameters. Its use in disease documentation produced statistically testable data which may "lead to new insights" into the problem (35). Point counting methods are fast and reliable, and require little hardware. The use of photomicrographs, instead of making measurements from prepared slides, lends itself to easy information storage and retrieval. However, the resolution of the cell types using a slide viewer was sometimes difficult, especially in chlorotic tissues where cell collapse and artifacts appeared similar. In this case, having the original glass slide available was helpful, if not essential.

In the chlorotic tissues, chlorenchyma collapse was apparent. This could account for the chlorosis associated with this disease. Maintenance of achlorophyllous tissue, *e.g.*, epidermis, bundle sheath, and vascular tissue, is consistent with the observations that the lesions became translucent, and necrosis of the entire tissue didn't occur until later. Of interest was the apparent increase in the volumetric proportions of the lower epidermis and vascular

cells (table 3). Possible explanations are a) the random-grid pattern tended to "match up" with the sinuous layers formed from the collapse of mesophyll cells, b) the tissue collapse resulted in a compaction of cells from all directions, not just from top to bottom, resulting in a disproportionately larger chlorotic area being sampled compared to the healthy tissues, and/or c) since the plants were grown in a well hydrated environment, the loss of mesophyll and water storage cells resulted in a water surplus and, if elastic enough, the remaining tissues swelled.

The first explanation is contrary to the concept of randomness but could have occurred. The second wasn't observed during the sampling procedures; the collapse of the lesion didn't appear to cause a crinkling or rippling of the remaining tissues, but at the macroscopic level, this could have been deceiving. The final possibility is the author's preference. Peanuts are known to have a well hydrated cellular environment and greater adaxial surface conductance (23). As water was taken up by tissues in the lesioned areas, living cells (epidermal, water storage cells, and some vascular cells) might swell. The upper epidermal tissues could lose more water to evapotranspiration, explaining their volume decrease.

Tannin cell decrease was markedly noticed in lesioned tissues. As these cells degrade, leakage of tanniferous substances into the surrouding apoplastic regions could

cause problems. Previous histochemical studies could not demonstrate that these cells contained tannins, but smaller "pockets" of tannins were found dispersed through many mesophyll cell types (9,10).

Overall, chlorosis, associated with chlorenchyma collapse, to necrosis, as non-chlorenchymatous tissues degrade, traces the disease process anatomically. The cause of the disorder is unknown, and possible mechanisms of tissue collapse are a matter for conjecture. Further research on this problem should probably concentrate on histochemically localizing deleterious substances and, biochemically, determining if high concentrations occur at these sites to result in the damage observed.

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APPENDIX

TABLE I

TOTAL NUMBER OF POINTS COUNTED AND THE MEAN COUNT PER SECTION

Tissue Component	Healthy		Chlorotic	
	Total	Mean±S.E.	Total	Mean±S.E.
upper epidermis	438	11.0±0.3	398	10.0±0.3
lower epidermis	327	8.2±0.3	397	9.9±0.4
palisade parenchyma	1040	26.0±0.8	320	8.0±0.4
spongy parenchyma	714	17.8±0.5	215	5.4±0.4
collapsed chlorenchyma	0		232	5.8±0.5
water storage cells	533	13.3±0.5	302	7.6±0.5
collapsed water storage cells	9	0.2±0.1	80	2.0±0.2
tannin cells	83	2.1±0.3	9	0.2±0.1
vascular cells	236	5.9±0.3	271	6.8±0.6
bundle sheath and extension	861	21.5±0.9	548	13.7±0.6
guard cells	93	2.3±0.2	89	2.2±0.2
intercellular space	725	18.1±0.7	247	6.2±0.3
total	5059	126.5±1.9	3018	77.7±1.4

S.E.= standard error of the mean (n=40)

TABLE II

PERCENT VOLUME OF EACH TISSUE COMPONENT
(VOLUME COMPONENT / VOLUME TOTAL)

Tissue Component	Healthy±S.E. (%)	Chlorotic±S.E. (%)
upper epidermis	8.8±0.3	13.1±0.6
lower epidermis	6.5±0.3	12.7±0.6
palisade parenchyma	20.4±0.7	10.4±0.8
spongy parenchyma	14.1±0.5	6.9±0.7
collapsed chlorenchyma	0	7.5±0.8
water storage cells	10.6±0.7	9.6±1.0
collapsed water storage cells	0.2±0.1	2.6±0.4
tannin cells	1.6±0.7	0.3±0.1
vascular cells	4.7±0.4	8.5±0.9
bundle sheath and extension	16.9±0.8	17.4±0.9
guard cells	1.9±0.3	3.0±0.9
intercellular space	14.2±0.6	8.0±0.5
total	99.9	100.0

S.E.= standard error of the mean (n=40)

TABLE III

VOLUME REMAINING AS A PERCENT OF CONTROL
(VOLUME CHLOROTIC / VOLUME HEALTHY *100)

Tissue Component	Volume (%)	
upper epidermis	90.8	
lower epidermis	121.4	(+21.4)
palisade parenchyma	30.8	
spongy parenchyma	30.1	
water storage cells	56.7	
tannin cells	10.2	
vascular cells	114.8	(+14.8)
bundle sheath and extension	63.7	
guard cells	95.7	
intercellular space	34.1	
total (by volume)	61.4	
(by occular micrometer height)	61.8	

VITA 2

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Thesis: VOLUMETRIC CHANGES AND THEIR IMPLICATIONS TO TISSUE COLLAPSE IN A LEAFSPOT IN AN *ARACHIS* INTERSPECIFIC HYBRID

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