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# Genetic and cytological studies of the necrotic leaf-spot mutation in cotton

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# GENETIC AND CYTOLOGICAL STUDIES OF THE NECROTIC LEAF-SPOT MUTATION IN COTTON

by

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## APPROVAL BY THESIS DIRECTOR

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# ABSTRACT

The cotton (*Gossypium hirsutum* L.) necrotic leaf-spot mutation is due to a single recessive gene. Cytologically the necrotic leaf-spot mutant affects the development and necrosis of leaves associated with the changed staining pattern, contraction and collapse of palisade and spongy parenchyma cells sequentially. The scanning electron microscope revealed that the stomata are also involved or affected by necrosis. The mutation exhibits a slightly deleterious effect on pollen viability, but not on the viability of the embryo.

# INTRODUCTION

Necrosis is usually recognized as symptoms of nutrient deficiencies (Sprague, 1964; Salisbury and Ross, 1985), phytopathogenic infection (Wheeler, 1981; Lamari and Bernier, 1989) or viral invasion (Cho and Chung, 1977; Kwon and Oh, 1980). Genetic necrosis has been reported in hybrids of wheat, rye and triticale due to the complementary reaction of two dominant genes (Hermsen, 1963; Ren and Lelley, 1988), and the incompatibility in crosses between plant species (Phillips and Merritt, 1972; Scoles, 1985).

Heritable leaf necrosis in cotton has not been documented. In 1977, a necrotic leaf-spot mutation was originally discovered by Dr. L. S. Stith of the University of Arizona in a maintenance plot of Delcott 277 R, which segregated at a ratio of 12 necrotic : 8 normal plants. Delcott 277 R is a *G. hirsutum* varietal type with the pollen fertility restorer gene and the cytoplasm of *G. harknessii* Brandy., $D_{2-2}$. Progeny of this line, designated as AG272, will be referred to in the text and the tables as necrotic-*G. harknessii* cytoplasm or $D_{2-2}$ cyto. Self-pollinated progenies of necrotic plants expressed the necrotic leaf-spot symptoms in 1978 and later (Endrizzi and Ray, personal communication; detailed information in Figure 1A). Endrizzi successfully transferred this mutation into a *G. hirsutum* L. line with *G. hirsutum* cytoplasm (Figure 1B). Progeny from this transfer, designated AG273, will be referred to in the text and the tables as necrotic-*G.hirsutum* cytoplasm or hirs cyto. In a preliminary genetic test with these materials Endrizzi (personal communication) observed the segregation patterns suggesting that the ratio of normal vs. necrotic plants may depend on the cytoplasmic background of the female parent in the initial cross.

The necrotic leaf-spot mutation causes the gradual death of infected areas of cotton leaves, with symptoms starting just before flowering. The first sign of necrosis is the appearance of a tiny dark spot on the leaf surface. The central area of the spot becomes brown or gray, resulting in a dark ring around the edge of the spot as the symptom develops. The development of the symptom is heterogeneous, some affected areas may increase in size very rapidly, and other affected areas may show very little increase in the spot size, i.e. little or no discernable increase in the size. Necrotic plants do survive, although the symptoms become more severe throughout the growing season.

A. **AG272** ($D_{2-2}$ cyto, necrotic):

Delcott 277 R (self-pollinated)

8 normal

12 necrotic(9-9-4-77) S/1 ——> J16-B-5-78 S/2 (all necrotic) ——> A18A-6-79 S/3 (necrotic) ——> H16B-2-80 S/4 (necrotic) ——> F11-81 Bulk S/5 (necrotic) ——> F20B-82 Bulk S/6 (necrotic) ——> M2A-86 Bulk S/7 (necrotic) ——> B6-19-87 S/8 (necrotic) ——> B10C-5-89 S/9 (necrotic) ——> F17-90 (necrotic)

B. **AG273** (hirs cyto, necrotic):

F20A-82[TM1 S/36 X F11-81($D_{2-2}$ cyto, necrotic)]$F_1$ X F20B-82($D_{2-2}$ cyto, necrotic) ——> F30-5-83(normal) Backcross ——>J8-2-84$F_1$S[=(F30-5-83 X AG272), normal] ——> J13-7-85 S/2(necrotic) ——> MIA-1-86 S/3 (necrotic) ——> B4-2-87 S/4 (necrotic) ——> B10B-2-89 S/5 (necrotic) ——> F16-90 (necrotic)

Figure 1. Pedigrees for the parent-lines carrying the necrotic leaf-spot mutation used in this study ($D_{2-2}$ cyto = *G. harknessii* cytoplasm; hirs cyto = *G. hirsutum* cytoplasm; $F_1$ = the first filial generation of the cross; TM1 = Texas Maker 1; S/N indicates the generation (N) of self-pollination).

The necrotic leaf spot mutation is not lethal to plant and was originally associated with some male sterility, due perhaps to the cytoplasmic background. The study of this mutation might provide an approach to understand the effect of male sterility in cotton (Myer, 1971a, 1971b, 1975). In addition, this mutation might be utilized as a marker for screening programs in the development of male sterile lines. More importantly, the study of this mutation will provide some basic understanding of genetic necrosis in cotton, which might be used as a tool for investigations in the fields of physiology, genetics, cytogenetics and molecular biology.

The objectives of this research were to (1) investigate the inheritance pattern of the necrotic leaf-spot mutation; (2) determine whether the necrosis mutation is influenced by cytoplasmic or other nuclear factors; (3) compare normal and necrotic tissues cytologically; (4) ascertain whether the necrosis mutation has deleterious effects on the viability and fertility of gametes; and (5) collect descriptive data about this mutation.

# LITERATURE REVIEW

Necroses are generally classified into three categories according to their origin: (1) nutrient deficiencies; (2) phytopathogenic or viral infections; and (3) genetic factors. Necrosis due to phytopathogenes has received most of the research attentions, since it is a known phenomenon causing reduction in crop yields. The degree of damage due to necrosis is varied dependent upon its source, the symptom severity and the damaged parts of the crop. In a severe case, the whole crop can be destroyed, as in the wheat epidemic due to *Pyrenophora tritici-repentis* on the Canadian Prairies in 1974 (Tekauz, 1976). Most necroses, however, occur on the leaves and reduce crop yields significantly, due to a reduction in the total photosynthetic rate (Horsfall and Henberger, 1942; Large, 1958; American Phytopathogical Society, 1964).

Necroses due to nutrient deficiencies have been clearly described. Deficiencies of potassium, zinc or manganese might cause necrosis by affecting either constituent elements for cells or accessary factors for enzyme activities (Sprague, 1964; Gauch, 1972; Hewitt and

Smith, 1975; Vallee, 1976). As stated above, although the most vigorous research area concerning necrosis is on aspects of phytopathology and toxicology, the genetic aspects of pathogenic necroses have been researched, and the genetic aspects of necroses of the host and/or pathogen can provide the strongest evidence for toxin involvement (necrotic symptom) in diseases (Yorder, 1980; Scheffer, 1983).

## Genetic Studies of Necrosis

The researches on genetic necroses mainly focus hybrid necroses in wheat, rye, triticale and cotton. Hybrid necrosis is a physiological disorder leading to premature and gradual death of organs or tissues in hybrid plants. This phenomenon was found frequently by wheat breeders, and its genetics has been extensively studied (Hermsen, 1963; Zeven 1966, 1981). The earliest studies in wheat were carried out by McMillan (1936) and Kostyuchenko (1936). McMillan called this kind of necrosis, "*firing*", and indicated that the phenomenon was heritable. Kostyuchenko suggested that the necrosis was caused by the complementary action of two genes, $Ne_1$ and $Ne_2$, when dominant alleles at both genes were present. Caldwell and Compton (1943) later reported what they termed "*progressive necrosis*"--- a necrosis in wheat which continues progressively from the tips of leaves to their bases, and from the older to the younger leaves. So far, three alleles of $Ne_1$ and five of $Ne_2$ have been identified (Zeven, 1981), and different allelic combinations cause various degrees of necroses in wheat hybrids (Hermsen, 1963). Both genes are found in the B genome, with the $Ne_1$ locus on the long arm of chromosome 5B (Tsunewaki, 1960; Zeven, 1972), and the $Ne_2$ locus on the short arm of 2B (Tsunewaki, 1960; Zeven, 1972; McIntosh, 1973). The $Ne_1$ and $Ne_2$ alleles appear frequently in hexaploid wheat, but only $Ne_1$ has been detected in tetraploid wheat (Nishikawa, 1967), with over 5500 hexaploid and tetraploid wheat lines and cultivars being tested (Zeven, 1981; Pfeffer and Zeller, 1987).

In triticale, the necrosis has been detected (Gregory, 1973; Gupta and Priyadarahan, 1982; Behl and Maherchandani, 1983). By crossing hexaploid and octoploid triticales, $Ne_2$ can be brought together with $Ne_1$ just as in the crosses between hexaploid and tetraploid wheats. Jung and Lelley (1985) discovered the presence of a gene in a line of inbred rye which caused necrosis in triticale by the interaction with the $Ne_1$ gene of wheat. Concurrently, Scoles (1985) found another gene in a different rye inbred line which led to necrosis in crosses with a hexaploid wheat carrying *Ne*$_2$. Ren and Lelley (1988) verified that both necrosis genes exist in diploid ryes, and cause necrosis in rye hybrids by dominant complementary interaction; and they further (1989) confirmed that these genes can also cause necrosis in triticale by complementary action with either *Ne*$_1$ or *Ne*$_2$ of the wheat B genome. The genes in rye were named *Ner*$_1$ and *Ner*$_2$ according to their correspondence to the two wheat genes.

Hybrid necrosis occurs in the interspecific progenies of several cotton species as well, it is especially prevalent in crosses involving the two D-genome species *Gossypium klotzschianum* Anderss. and *G. gossypioides* (Vlbr.) Standl. A majority of the interspecific hybrids of *G. klotzschianum* result in the embryonic or seedling necrosis caused by an incompatibility in the interspecific crosses. The embryonic or seedling necrosis is explained as a lethal symptom of incompatibility, which is controlled by a major gene and modified by a series of minor genes. This model has been found to work in natural tetraploid *G. hirsutum* L. as well as in diploid cottons (Gerstel, 1954; Phillips, 1962, 1963).

Most crosses of *Gossypium davidsonii* Kell. with various diploid and tetraploid taxa of *Gossypium* result in embryonic necrosis and moribundity early in development. A few other hybrids, however, germinate normally, but become necrotic and die within days, or at most, weeks. Lee (1981) confirmed that the lethality is caused by the complementary interaction of the lethal factor $Le^{dav}$ from *G. davidsonii* ($D_3$) with $Le_1$ and $Le_2$ from *G. hirsutum* and *G. barbadensa*, which were identified by him as type-2 lethality (germination appeared normal, but the seedlings became necrotic within three or four days) and type-3 lethality (germination was normal and the seedlings survived for weeks or longer) respectively.

Rooney and Stelly (1989) reported that cotton zygotes bearing a $Le_2^{dav}$ alleles in combination with $Le_1$ and/or $Le_2$ undergo a physiological reaction resulting in severe embryonic necrosis before seedling emergence. The necrotic development is a result of the cumulative dosage of alleles $Le_1$ and $Le_2$ in the presence of $Le_2^{dav}$.

## Cytological and Physiological Studies of Hybrid Necrosis

The workers started to explore the physiological changes involved in hybrid necrosis at early 70's. However, so far the physiological mechanism of the necrosis is not clear. They have tried to correlate hybrid necrosis with the change in chemical content in necrotic plants, especially in wheat necrosis, however, no conclusive data have been obtained (Zeven, 1981).

Using chemical (folin) and histochemical (toluidine blue) tests, Phillips and Reid (1975) observed that phenolics were very conspicuous in and near necrotizing cells of the interspecific hybrids involving *Gossypium klotzschianum*, however, they found no correlation between necrosis and two groups of primary metabolites (amino acids and organic acids) in the hybrid leaves. They also discovered that some hybrids produced two phenotypes: embryonic and seedling necroses. Histologically, the necrotic symptoms appeared as necrotic cells and tumors 10 to 15 days after fertilization in embryonic necrosis, and at the first true-leaf stage in seedling necrosis. The ultrastructural studies of cell necrosis in seedlings showed that the first subcellular abnormality is the degeneration of the inner membrane of the envelope and cristae of the mitochondria. Mitochondrial degeneration is essentially completed before other organelles show any evidence of structural aberrations.

# MATERIALS AND METHODS

## Genetic Materials

The genetic materials used in this study were provided by Dr. John E. Endrizzi (Professor Emeritus, Department of Plant Sciences, University of Arizona). In 1989, the materials consisted of four $F_2$ and nine backcross populations (Table 1). The pedigrees of the parent-lines used in the crosses are diagramed in Figure 1. Based on their cytoplasmic types, the necrotic lines were classified into two categories: one with $D_{2-2}$ cytoplasm, and the other with *G. hirsutum* cytoplasm. The *G. hirsutum* L. genetic standard, Texas Marker 1, was included in the experiments as the normal or non-necrotic test line, and coded as TM1. The genetic studies of $F_2$ and backcross populations were conducted at the University of Arizona Campus Agricultural Center in 1989. $F_1$ populations were renewed in 1990 (the materials listed in Table 2). Normal cultural practices were employed in the field preparation, planting and management. Seeds of hybrids were sowed on April 27 and 28 of each year.

Table 1. Genetic materials† used in 1989

| Crosses | Types of the Crosses |
|---|---|
| A6-**-87$F_1$S§ | (hirs cyto‡ X TM1++)$F_1$S |
| A6-**-87$F_1$ X B4-87 | (hirs cyto X TM1)$F_1$ X hirs cyto |
| B4-**-87 X A6-87$F_1$ | hirs cyto X (hirs cyto X TM1)$F_1$ |
| B1-**-87$F_1$S | (TM1 X hirs cyto)$F_1$S |
| B1-**-87$F_1$ X B4 or 5-87 | (TM1 X hirs cyto)$F_1$ X hirs cyto |
| B5-**-87 X B1-**-87$F_1$ | hirs cyto X (TM1 X hirs cyto)$F_1$ |
| | |
| B2-**-87$F_1$S | ($D_{2-2}$ cyto+++ X TM1)$F_1$S |
| B2-**-87$F_1$ X B6-87 | ($D_{2-2}$ cyto X TM1)$F_1$ X $D_{2-2}$ cyto |
| B3-**-87$F_1$S | (TM1 X $D_{2-2}$ cyto)$F_1$S |
| B6-**-87 X B3-87$F_1$ | $D_{2-2}$ cyto X (TM1 X $D_{2-2}$ cyto)$F_1$ |
| | |
| B3-**-87$F_1$ X B4-87 | (TM1 X $D_{2-2}$ cyto)$F_1$ X hirs cyto |
| C1-**-87 X B1-87$F_1$ | $D_{2-2}$ cyto X (TM1 X hirs cyto)$F_1$ |
| B2-**-87$F_1$ X B4-87 | ($D_{2-2}$ cyto X TM1)$F_1$ X hirs cyto |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.
** the plant number is omited.
§ $F_1$S = self-pollination of the first filial generation of the cross.
‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.

Table 2. Genetic materials[†] used in 1990

| Genetic Materials | Types of the Genetic Materials |
|---|---|
| (B10B-2-89 X TM1)$F_1$[§] | (hirs cyto[‡] X TM1[++])$F_1$ |
| (TM1 X B10B-2-89)$F_1$ | (TM1 X hirs cyto)$F_1$ |
| (B10C-5-89 X TM1)$F_1$ | ($D_{2-2}$ cyto[+++] X TM1)$F_1$ |
| (TM1 X B10C-5-89)$F_1$ | (TM1 X $D_{2-2}$ cyto)$F_1$ |
| Parent-line (B10B-2-89) S/5[#] | (hirs cyto) S/5 |
| Parent-line (B10C-5-89) S/8 | ($D_{2-2}$ cyto) S/8 |
| Standard TM1 S/44 | (hirs cyto) S/44 |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.
§ $F_1$ = the first filial generation of the cross.
‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.
# S/N = generation (N) of self-pollination.

## Classification of Normal and Necrotic Plants

In 1989, necrotic symptoms started at blooming. A plant was classified as necrotic if any small or large necrotic spot(s) appeared on the leaves. Otherwise the plants were categorized as normal. The problem of distinguishing the necrotic leaf-spot mutation phenotype from other necroses will be discussed later under the "Description of Necrotic Symptoms" in the "Results and Discussion". Genetic segregations were scored in 1989, and the data (APPENDIX A) subjected to Chi-square ($X^2$) analysis. Two of the crosses were excluded in the results because of the contamination indicated by curly leaf and the unsuccessful crossing: ($D_{2-2}$ cyto X TM1)$F_1$S and $D_{2-2}$ cyto X (TM1 X hirs cyto)$F_1$.

## Size Frequency of Necrotic Spots

This experiment was carried out in 1990, and was limited to necrotic parent-lines. One hundred leaves were randomly selected from B10B-2-89 and B10C-5-89 (Table 2). Necrosis was scored according to spot diameter. The following ratings were used in the studies:

| | |
|---|---|
| tiny spot : | 1 to 2 mm diameter; |
| small spot: | 3 to 4 mm diameter; |
| large spot: | 5 to 6 mm diameter; |
| extra large spot: | >7 mm diameter. |

On each leaf, the number of necrotic spots was noted and rated as above.

## Evaluation of Pollen Viability

The procedure for evaluation of pollen viability was based on the Modified Fluorochrome Reaction (FCR) method of Heslop-Harrison (Heslop-Harrison and Heslop-Harrison, 1970; Gwyn and Stelly, 1989). This procedure evaluates pollen viability by determining the presence and the activities of several esterases known to be essential for normal gametophytic function. The esterases that are associated with fully functional cell walls hydrolyze the fluorescein diacetate, causing it to become photo-excitable at ultra-violet wavelengths. Pollen from seven lines (four $F_1$ progenies involving either necrotic parent, two necrotic parent-lines and TM1, Table 2) were evaluated in the summer, 1990, on four dates (July 13, 21, August 4 and 23). Each line had two replications per date, each replication included duplicated slides for pollen evaluation.

Stock solution-1 (SS-1) consisted of 1.75 M sucrose, 3.23 mM $H_3PO_4$, 3.05 mM $Ca(NO_3)_2$, 3.33 mM $MgSO_4$, 1.98 mM $KNO_3$. The high sugar and salt concentration was to prevent osmotic bursting and induce maximal fluorochrome reaction. The stock solution-2 (SS-2) consisted of 7.21 mM fluorescein diacetate dissolved in pure acetone. The stock solutions were stored in a cold room (4 to 8°C). The working solution was freshly prepared hourly by incrementally adding 10 to 15 drops of SS-2 into 10 ml SS-1 until the composite solution was slightly milky.

Flowers were collected at anthesis. Petals were removed, pollen generously dusted on a glass slide, 2 to 3 drops of working solution added, and a coverslip applied. In order to allow fluorochrome uptake and the reaction to occur, the slides (consisting of 5 to 7 flowers per slide) were counted about 5 minutes after the preparation, with an Olympus Inverted Microscope (IMT-2) equipped with epifluorescence, barrier filter of 520 nm and exciter filter of 400-490 nm (blue), at a magnification of 100X.

The statistic analysis was conducted after data (APPENDIX B) transformation [arc sine $(\sqrt{\text{pollen viability(\%)}})$] and homogeneity test of variance (Gomez and Gomez, 1984).

The analysis of variance followed the model for a Randomized Complete Block experiment over years (or dates) (Gomez and Gomez, 1984). The comparisons among the lines were conducted through the Duncan Multiple Range Tests (DMRT). All the data transformation, the homogeneity test of variances, the analysis of variances and DMRT were computed using the SAS program (version 6.03) (SAS Institute, Inc., 1989). In order to simplify the statistic analyses, the ($D_{2-2}$ cyto X TM1)$F_1$ progeny and the self-pollinated $D_{2-2}$ cyto parent-line were not included in the statistic analysis since they have different cytoplasmic background.

## Evaluation of Embryo Viability

Cotton seeds were collected from the mature cotton bolls. The seeds were delinted in concentrated sulfuric acid ($H_2SO_4$) for 4 to 5 minutes until all lint disappeared, rinsed with tap water, treated in 85°C water for 1.5 minutes to break dormancy, then immediately cooled in tap water. The seeds were placed between two pieces of water-soaked germination paper. The germination papers were wrapped and placed into 1000 ml beakers containing about 100 ml water, with the tip ends (micropyler) of the seeds facing the water.

The beaker was covered, and stored at the optimal germination temperature of 25 to 28°C. The germinated seeds were counted after 48 to 60 hours.

The data (APPENDIX C) were subjected to analysis after data transformation [arc sine ($\sqrt{\text{embryo viability(\%)}}$ )] (Gomez and Gomez, 1984). The analysis of variance followed the model for a Complete Randomized Design (Gomez and Gomez, 1984). All data transformation and the analysis of variance were computed using the SAS program (version 6.03) (SAS Institute, Inc., 1989).

## Light and Scanning Electron Microscopy

Leaves with different size necrotic spots were collected, sliced into segments of 1 cm in length and 0.1 to 0.3 cm in width, and fixed in chilled 5% glutaraldehyde buffered with 0.1 M phosphate (pH=7.2). Fixation was carried out in a cold room (4 to 8°C) for 16 hours. The samples were rinsed with cold 0.1 M phosphate buffer (pH=7.2), the buffer was changed 3 to 4 times a day for 3 days. The post-fixation was conducted in 2%(w/v) $OsO_4$ buffered with 0.1 M phosphate, kept in a cold room overnight, then rinsed with cold 0.1 M phosphate 3 to 4 times. The dehydration was conducted through a graded ethanol series [1X 70%(v/v) ETOH, 15 minutes; 2X 95% ETOH, 20 minutes each; 3X 100% ETOH, 30 minutes each]. The samples were ready either for infiltration and embedding or critical point drying and gold sputtering.

The fixed samples for light microscopy were first infiltrated in a 50/50 mixture of pure ethanol and Spurr resin (Spurr, 1969), covered in a fume hood for 4 hours, then in pure Spurr resin uncovered in a fume hood overnight. Spurr resin consists of 6.5 ml nonenylsuccinic anhydride, 2.5 ml ERL (a cycloaliphatic diepoxide), 1.5 ml DER (diglycidyl ether of polypropylene glycol) and 0.1 ml dimethylaminoethyl. The infiltrated samples were transferred to a plastic mold filled with pure Spurr resin. The polymerization of the Spurr resin was conducted in a dry oven at 60$^{o}$C for 12 to 24 hours until they were hardened. The embedded sample was sectioned with a Reichert-Jung microtome (Ultracut E). The sections were 1 um in thickness, stained with uranyl acetate [1%(w/v) aqueous solution] at room temperature for 10 minutes, and observed under a light microscope (Olympus Inverted Microscope, IMT-2).

For scanning electron microscopy, the fixed samples were mounted on aluminium stubs using an organic resistance tape, further dried through the critical point of acetone (-20$^{o}$C) for 20 minutes, and gold-sputtered. The samples were scanned with a scanning electron microscope (JEOL, Ltd., I.S.I. DS-130).

# RESULTS AND DISCUSSION

## Description of Necrotic Leaf-spot Symptoms

The necrotic symptoms start just before the blooming period of cotton. These symptoms only appear on the leaves, with no necrotic spot having been detected on other organs. It is difficult to detect symptoms at the early stage of necrosis. Necrosis is first seen as a tiny dark spot on the leaf surface (Figure 2). Spots do not appear on veins, just on intervein tissues, with few leaves showing necrotic symptoms at this early stage. Symptoms appear first on the lower leaves, and newly-formed leaves have not been observed with necrotic symptoms. Necrotic spots at this early stage possess the following characteristics: small (size range:1 to 4 mm in diameter), dark, round in shape, and quite uniform. The early stage lasts 1 to 1.5 months.

During the middle stage of necrosis, the necrotic symptoms are quite obvious, with 10 to 20% of mature leaves showing necrotic spots. This stage is characterized by large necrotic spots. The central areas of the spots become dark brown or grey, completely dried-out, and a sharp dark

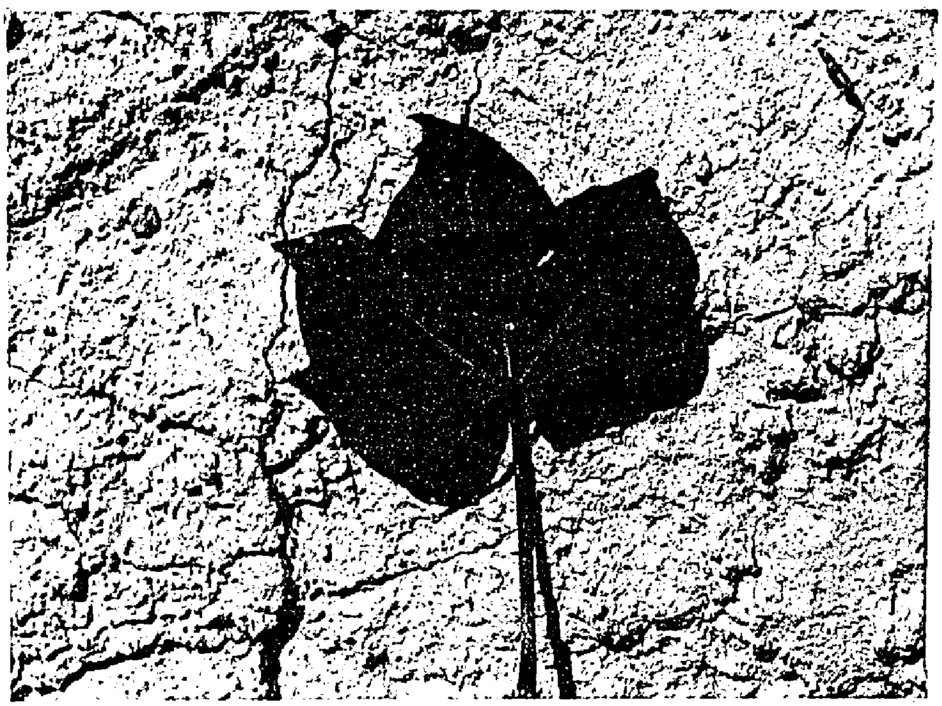

Figure 2. Early stage of necrotic leaf-spot, showing three tiny dark necrotic spots.

ring is formed around the edge of the necrotic spot (Figure 3). The necrotic symptoms at this stage may potentially influence the vegetative and reproductive growth of cotton plants. The middle stage lasts about 1 month.

The late stage of the necrotic symptoms occurs during the maturing period of cotton bolls. Most of the mature leaves are severely damaged, the sizes and shapes of the necrotic spots are quite irregular at this period (Figure 4). The spot keeps expanding with more and more of the inner area becoming dark brown or grey, and the dark surrounding ring expands as the spot develops. Necrotic symptoms damage 30 to 50% of the leaf area. It is easy to observe the impact of the necrotic symptoms on the growth of cotton plants at this stage. The plants with severe necrotic symptoms are smaller in size when compared with normal plants, and the lower leaves fall off. However, the newly-formed leaves are still normal in appearance. The appearance, size and shape of necrotic cotton plants show a great heterogeneity at the late stage.

The three necrotic stages were classified by the most apparent symptoms during each period. The development of necrosis is quite heterogeneous, with symptoms of all three stages detected on a necrotic plant. One point is certain,

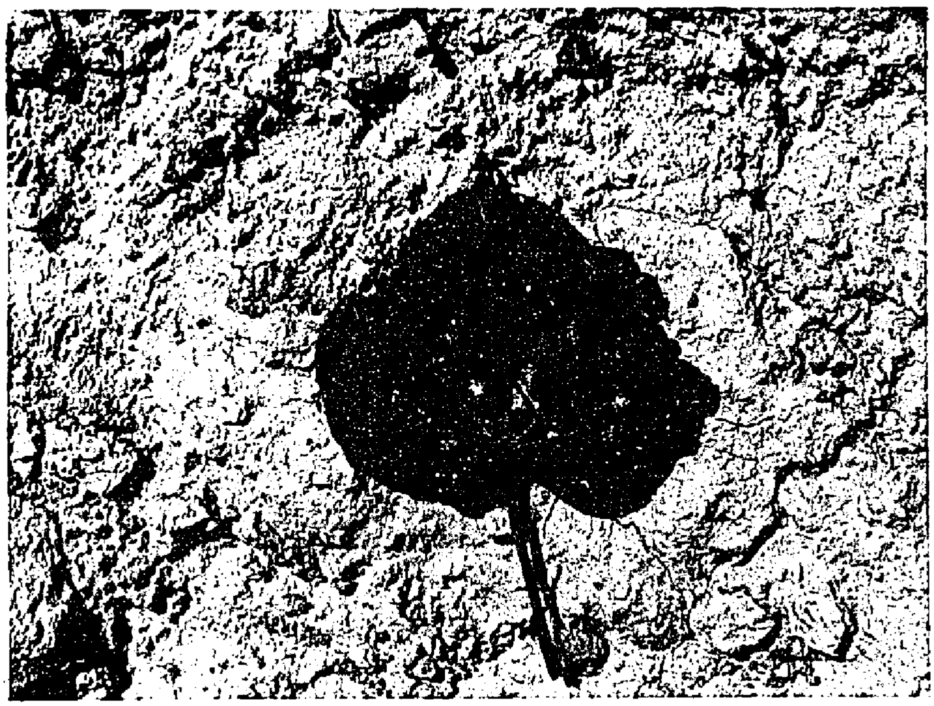

Figure 3. Middle stage of necrotic leaf-spot, showing the increased spot size, the inner brown or grey area inside and the dark ring around the edge of the necrotic spots.

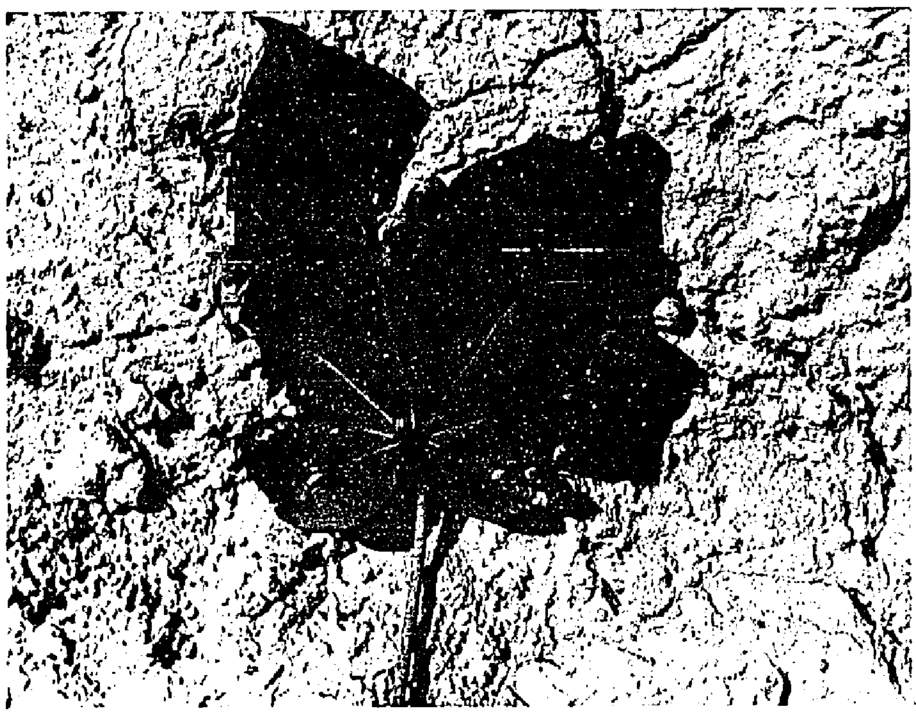

Figure 4. Late stage of necrotic leaf-spot, showing severe damage to the leaf tissue.

the necrotic symptoms appear during the maturing stage of cotton leaves.

This leaf-spot necrosis is distinguishable from the other necroses caused by phyto-pathogenic factors in cotton: by the 1) the developmental rate of necrosis, 2) the sustaining period of necrosis, 3) the characteristics of the necrotic spot, and 4) its impact on the growth of cotton plants (Wheeler, 1969; Prentice, 1972; Weber, 1973). Compared with phytopathogenic necroses, the leaf-spot necrosis slowly develops; the symptoms last for a whole growing season, and the necrotic leaf-spot development is synchronized with the maturing of the leaves. The unique characteristic of the cotton necrotic leaf-spot is the dark ring around the edge of the spots during the late-early or early-middle stage of necrosis (spot size 4 to 5 mm in diameter). The influence of the mutant on plant growth is not detectable within a short period (3-4 weeks); however, after most leaves show symptoms, the impact can be observed on vegetative growth.

## Necrotic Leaf-spot Size and Frequency

The frequency of necrotic spots was investigated on three different dates during development (July 21, August 20 and September 21) in 1990. Based on four ratings (see the "Frequency of Necrotic Spots" in the "Materials and Methods"), the sizes of necrotic spots on 100 leaves randomly selected per sampling date from parent-lines were classified.

The data in Table 3 indicate that the number and size of necrotic spots were positively related to the rate of the development; tiny and small spots were most frequent at the early stage; and the proportion of large and extra large spots rised as necrosis proceeded to the middle and late stages. The studies confirmed that the occurrence of the necrotic leaf-spot was associated with a specific period of cotton growth (the maturing period of leaf); furthermore, the development of necrosis is dependent on the interaction of leaf expansion, climate and other factors.

## Genetic Studies of Necrotic Leaf-spot Mutation

### 1. Analysis of $F_1$ populations

$F_1$ populations were not available in 1989. However, in personal communication with Dr.J.E. Endrizzi, he indicated that all the $F_1$ populations of the crosses used in this study were normal in appearance. In 1990, $F_1$ seeds of reciprocal crosses were planted and no necrotic symptoms were detected

Table 3. Size frequency of necrotic leaf-spots*

| Classification of Spot Size | Sampling Date 7/21/90 | 8/20/90 | 9/21/90 |
|---|---|---|---|
| | ------ number of spots ------ | | |
| tiny spot (1-2mm in diameter) | 28 | 42 | 15 |
| small spot (3-4mm in diameter) | 44 | 67 | 56 |
| large spot (5-6mm in diameter) | 3 | 96 | 108 |
| extra large spot (>7mm in diameter) | 0 | 35 | 178 |
| Total | 75 | 240 | 357 |

* The frequency study was conducted on 100 randomly selected leaves per sampling date from the two necrotic parent-lines B10B-2-89 and B10C-5-89 respectively.

(Table 4). The self-pollinated parent-lines showed severe symptoms, and the standard cotton plants (TM1) were normal phenotypically.

Based on the results in Table 4, since the $F_1$ plants were non-necrotic, we can infer that the necrotic leaf-spot mutation is recessive to normal. The other point was that the non-occurence of the necrotic symptom in $F_1$ progenies was not related to the cytoplasms of female parent-lines. Thus, a conclusion can be drawn that an extranuclear gene is not involved in the necrotic leaf-spot mutation. Although the hypothesis of cytoplasmic inheritance of necrotic mutation was eliminated, the interaction between nuclear and cytoplasmic genes needed to be further tested in the $F_2$ populations.

2. Analysis of $F_2$ populations

The results (Table 5) of segregating populations firmly indicated that the necrotic leaf-spot phenotype is due to homozygosity of a recessive nuclear allele, as the mutation exhibited Mendelian segregation ratios of a single gene in the $F_2$ and backcross populations. Furthermore, the cytoplasmic background of the female parents did not affect the segregation ratios of the $F_2$ and backcross populations,

Table 4. Genetic data of parental and $F_1$ populations† from the crosses between TM1 and necrotic parents

| Types of Hybrid | Total | Observed Phenotypes | |
|---|---|---|---|
| | | Normal | Necrotic |
| | -------- Number of Plant ------- | | |
| (hirs cyto‡ X TM1++) $F_1$§ | 20 | 20 | 0 |
| (TM1 X hirs cyto) $F_1$ | 20 | 20 | 0 |
| ($D_{2-2}$ cyto+++ X TM1) $F_1$ | 19 | 19 | 0 |
| (TM1 X $D_{2-2}$ cyto) $F_1$ | 19 | 19 | 0 |
| Parent-line (hirs cyto) S# | 20 | 0 | 20 |
| Parent-line ($D_{2-2}$ cyto) S | 20 | 0 | 20 |
| Standard TM1 | 20 | 20 | 0 |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.
‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
§ $F_1$ = the first filial generation of the cross.
+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.
# S = generation (N) of self-pollination.

Table 5. Summary of the inheritance data for the necrotic mutant from segregating populations†

| Types of the Crosses | Total Plants | Phenotypes NOR* | NEC** | $X^2$ | P@ |
|---|---|---|---|---|---|
| | ------ | Number | ----- | | % |
| (hirs cyto‡ X TM1++) $F_1S$§ | 293 | 228 | 65 | 1.24 (3:1) | 0.30-0.20 |
| (hirs cyto X TM1) X hirs cyto | 62 | 38 | 24 | 3.16 (1:1) | 0.10-0.05 |
| hirs cyto X (hirs cyto X TM1) | 83 | 38 | 45 | 0.59 (1:1) | 0.50-0.30 |
| (TM1 X hirs cyto) $F_1S$ | 287 | 221 | 66 | 0.61 (3:1) | 0.50-0.30 |
| (TM1 X hirs cyto) X hirs cyto | 172 | 92 | 80 | 0.84 (1:1) | 0.50-0.30 |
| hirs cyto X (TM1 X hirs cyto) | 9 | 5 | 4 | 0.11 (1:1) | 0.95-0.90 |
| ($D_{2-2}$ cyto+++X TM1) X $D_{2-2}$ cyto | 94 | 53 | 41 | 1.54 (1:1) | 0.30-0.20 |
| (TM1 X $D_{2-2}$ cyto)$F_1S$ | 429 | 332 | 97 | 1.31 (3:1) | 0.30-0.20 |
| $D_{2-2}$ cyto X (TM1 X $D_{2-2}$ cyto) | 82 | 50 | 32 | 4.00 (1:1) | 0.05-0.02 |
| (TM1 X $D_{2-2}$ cyto) X hirs cyto | 37 | 17 | 20 | 0.24 (1:1) | 0.70-0.50 |
| ($D_{2-2}$ cyto X TM1) X hirs cyto | 108 | 61 | 47 | 1.82 (1:1) | 0.20-0.10 |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.
* NOR = normal plant, ** NEC = necrotic plant.
@ The value from R.A. Fisher and F. Yates, 1963. *Statistical Table for Biological, Agricultural and Medical Research* (6th edition) Table IV. Oliver and Boyd Ltd, Edinburgh.
‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
§ $F_1S$ = self-pollination of the first filial generation of the cross.
+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.

demonstrating that the hypothesis of the interaction between nuclear and cytoplasmic genes was disproved.

## Effect of the Mutation on Gametes

It was observed that the number of normal plants was frequently higher and the number of necrotic plants lower than expected. These small deviations did not influence the $X^2$ analysis for each cross significantly, but the deviations seemed unidirectional and quite homogeneous (Table 6). As indicated in Table 5, the $X^2$ analysis for each cross indicated no significant differences between the observed and the expected numbers, however, the $X^2$ analysis of the pooled numbers showed a significant deviation(Table 6). We suspected that the mutation may have small deleterious effect on the viability of the gametophytes. It was necessary to conduct further experiments on pollen and embryo viability to confirm this hypothesis.

Although mature cotton pollen often possesses a natural blue-green fluorescence, the intensity of the natural fluorescence is well below the intensity that is attained by the FCR method. The FCR method classifies the pollen

Table 6. Deleterious effect of the mutation on gametes

| Ratio | Total | Normal Phenotype | | | Necrotic Phenotype | | |
|---|---|---|---|---|---|---|---|
| | | observed | expected | deviation | observed | expected | deviation |
| | | Number of Plants | | | | | |
| 3:1 | 293 | 228 | 219.75 | +8.25 | 65 | 73.25 | -8.25 |
| 1:1 | 62 | 38 | 31.00 | +7.00 | 24 | 31.00 | -7.00 |
| 1:1 | 83 | 38 | 41.50 | -3.50 | 45 | 41.50 | +3.50 |
| 3:1 | 287 | 221 | 215.25 | +5.75 | 66 | 71.75 | -5.75 |
| 1:1 | 172 | 92 | 86.00 | +6.00 | 80 | 86.00 | -6.00 |
| 1:1 | 9 | 5 | 4.50 | +0.50 | 4 | 4.50 | -0.50 |
| 1:1 | 94 | 53 | 47.00 | +6.00 | 41 | 47.00 | -6.00 |
| 3:1 | 429 | 332 | 321.75 | +10.25 | 97 | 107.25 | -10.25 |
| 1:1 | 82 | 50 | 41.00 | +9.00 | 32 | 41.00 | -9.00 |
| 1:1 | 37 | 17 | 18.50 | -1.50 | 20 | 18.50 | +1.50 |
| 1:1 | 108 | 61 | 54.00 | +7.00 | 47 | 54.00 | -7.00 |
| total | 1656 | 1135 | 1080.25 | +54.75 | 521 | 575.75 | -54.75 |

$X^2 = 7.98^{**}$ (df=1, $P_{0.05}=3.84^{@}$, $P_{0.01}=6.64^{@}$)

** significant level at P = 0.01.
@ The value from R.A. Fisher and F. Yates, 1963. *Statistical Table for Biological, Agricultural and Medical Research* (6th edition) Table IV. Oliver and Boyd Ltd, Edinburgh.

viability and fertility based on two factors: variability in pollen morphology and fluorescence intensity. The largest pollen grains were fully engorged with starch, fluoresced bright luminescent green, and were considered fully viable (fertile). The nonviable category consisted of the following pollen types (Gwyn and Stelly, 1989): (1) small pollen but with bright fluorescence, (2) large or small pollen with dim fluorescence, (3) pollen with uneven fluorescence (lighter and darker areas), and (4) pollen without FCR fluorescence (but with a natural fluorescence). Pollen viability was evaluated on four dates (July 13, July 21, August 4 and 18) during the blooming period.

The analysis of variances (Table 7) indicated that a significant difference existed among dates, this result was similar to that reported by Gwyn and Stelly (1989). This was also confirmed by the changes of pollen viability in male sterile $F_1$ progeny of ($D_{2-2}$ cyto X TM1) and self-pollinated ($D_{2-2}$ cyto) parent-line during the flowering period (APPENDIX B). There were significant differences ($P = 0.01$) among the lines. Further comparisons among lines (Table 8) indicated that except for (TM1 X hirs cyto) $F_1$ progeny, the lines carrying the necrotic leaf-spot mutation had significantly ($P = 0.05$) reduced pollen viability. Although the pollen

Table 7. Analysis of variance of pollen viability after arc sine ($\sqrt{\text{pollen viability(\%)}}$) transformation

| Source of Variances | df | SS | MS | F | Tabular F[@] 5% | Tabular F[@] 1% |
|---|---|---|---|---|---|---|
| Date | 3 | 146.74 | 48.91 | 27.79** | 6.59 | 16.69 |
| Replication W/in date | 4 | 7.04 | 1.76 | | | |
| Lines | 4 | 503.11 | 125.78 | 5.71** | 3.26 | 5.41 |
| Lines X date | 12 | 264.16 | 22.01 | 2.49 * | 2.42 | 3.55 |
| Pooled Error | 16 | 141.48 | 8.83 | | | |
| Total | 39 | 1062.28 | | | | |

* significant level = 0.05, ** significant level = 0.01.

[@] The values from K.A. Gomez and A.A. Gomez, 1984. *Statistical Procedure for Agricultural Research* (2nd edition), Appendix E. John Wiley & Sons, Inc., NY.

Table 8. Comparisons of pollen viability(%) among the lines†

| Type of Hybrid | Average Pollen Viability♥ | DMRT♣ |
|---|---|---|
| | % | |
| (hirs cyto‡ X TM1++)$F_1$§ | 87.8 | AB♦ |
| (TM1 X hirs cyto)$F_1$ | 89.6 | BC |
| ($D_2$-2 cyto+++ X TM1)$F_1$ | 5.1 | ---* |
| (TM1 X $D_2$-2 cyto)$F_1$ | 86.9 | AB |
| Parent-line (hirs cyto)S# | 81.6 | A |
| Parent-line ($D_2$-2 cyto)S | 6.8 | ---* |
| Standard TM1 | 94.3 | C |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.

♥ Average of 4 dates (July 13, 21, August 4 and 18) with 2 replications.

♣ DMRT = Duncan Multiple Range Tests.

‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.

++ TM1 = Texas Maker 1.

§ $F_1$ = the first filial generation of the cross.

♦ Any two means indicated by the same letter are not significantly different at the 5% level of significance.

+++ $D_2$-2 cyto = necrotic-*G. harknessii* cytoplasm.

* Not included in the statistic analysis.

\# S = generation (N) of self-pollination.

viability of (TM1 X hirs cyto) $F_1$ progeny was not reduced significantly, it was still lower than that of TM1.

Embryo viability was evaluated with seeds collected in 1990. Seven lines, each with two replications (50 seeds per replication) were counted (APPENDIX B).

The analysis of variance showed that there were no significant differences among the lines for embryo viability (Table 9). The results indicated that the necrotic leaf-spot mutation did not greatly influence the embryo viability (Table 10).

## Cytological and Morphological Studies of Necrotic Leaf-spots

### 1. Cytological Studies

The cytological studies indicated that the early development of necrosis occurs in the palisade cells. The cells became changed in staining pattern and contracted in cell shape (Figure 5), which are concomitant with the dark spot during the early stage of necrosis or the dark ring around the edge of the necrotic spot during the middle or late stages of necrosis (Figures 2, 3 and 4). The spongy parenchyma cells start to change in staining pattern and

Table 9. Analysis of variance of embryo viability after arc sine ($\sqrt{\text{embryo viability(\%)}}$ ) transformation

| Source of Variances | df | SS | MS | F | Tabular F[@] 5% | 1% |
|---|---|---|---|---|---|---|
| Lines | 6 | 187.06 | 31.18 | 3.64 | 4.28 | 8.47 |
| Replications | 1 | 2.12 | 2.12 | 0.25 | 5.99 | 13.74 |
| Pooled Error | 6 | 51.44 | 8.57 | | | |
| Total | 13 | 240.62 | | | | |

[@] The values from K.A. Gomez and A.A. Gomez, 1984. *Statistical Procedure for Agricultural Research* (2nd edition), Appendix E. John Wiley & Sons, Inc., NY.

Table 10. Embryo viability(%) of the lines†

| Type of Hybrid | Average Embryo Viability♥ |
|---|---|
| | % |
| (hirs cyto‡ X TM1++ )$F_1$§ | 94.0 |
| (TM1 X hirs cyto)$F_1$ | 93.0 |
| ($D_2$-2 cyto+++ X TM1)$F_1$ | 95.0 |
| (TM1 X $D_2$-2 cyto)$F_1$ | 93.0 |
| Parent-line (hirs cyto)S# | 83.0 |
| Parent-line ($D_2$-2 cyto)S | 89.0 |
| Standard TM1 | 89.0 |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.
♥ Average of 2 replications.
‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
§ $F_1$ = the first filial generation of the cross.
+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.
# S = generation (N) of self-pollination.

contract in shape as palisade cells start to collapse. The brown or grey area inside the dark ring (Figures 3 and 4) are associated with the completely collapsed palisade and spongy parenchyma cells inside the leaf (Figure 6). The necrotic area typically occurs in the palisade and spongy parenchyma cells, whereas the cells of the vascular system are usually normal unless they are invaded by the expanding necrotic palisade and/or spongy parenchyma cells. The epidermis of the leaf stays a normal rigid structure, even if the cells inside the leaf are completely destructed (Figure 6).

2. Scanning Electron Microscopy of Necrotic Leaf-spots

The normal epidermis of leaves has fully expanded epidermal cells and open stomata (Figure 7). The necrotic spot is characterized with the collapsed epidermal cells and closed stomata, even though the necrotic epidermis is intact (Figure 8). The closed stomata seem to be a natural response to the loss of water inside leaf, this may be the reason why necrotic leaf-spots develop so slowly. The collapsed but intact epidermal cells are quite similar to the light microscopic observations of the epidermal cells in the inner brown or grey area of the necrotic spot (Figure 6). However, the inner brown or grey area of the necrotic spot is quite brittle and easily popped-up (Figure 9).

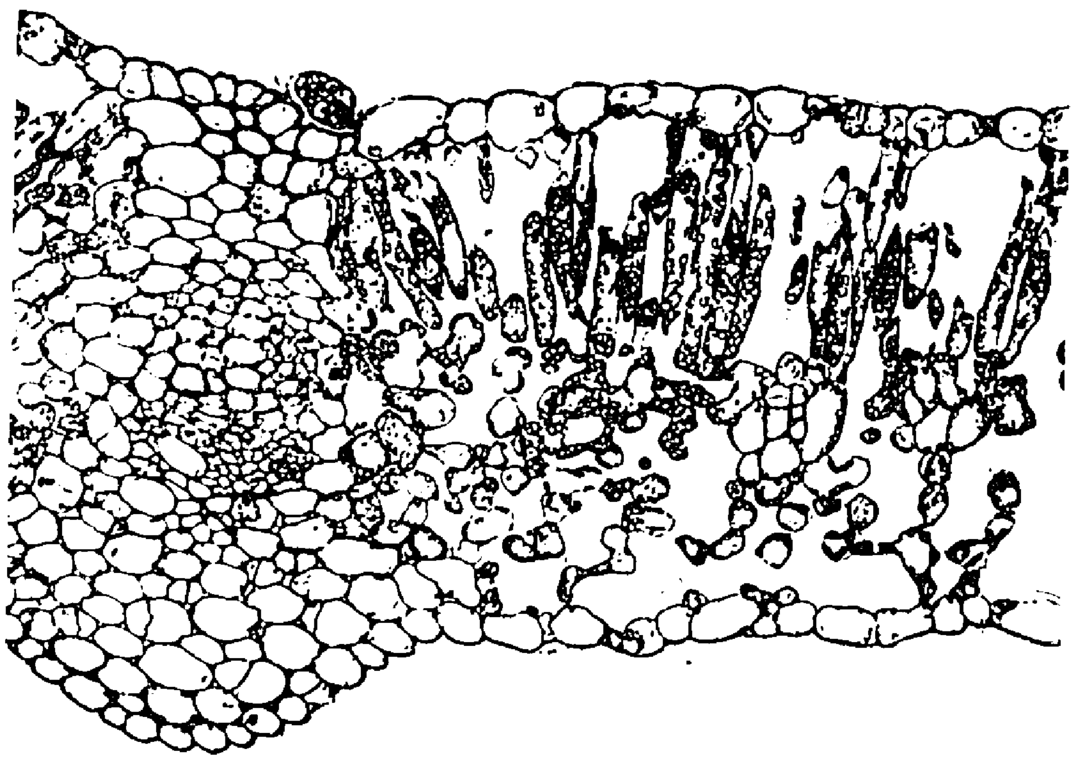

Figure 5. Leaf transection of the interface between normal and necrotic tissues, showing contracted palisade cells, 125 X.

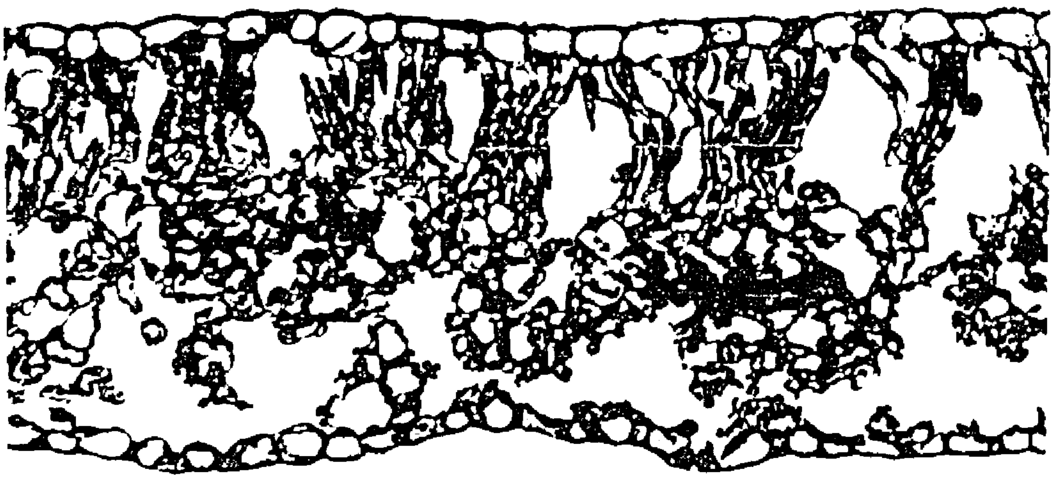

Figure 6. Leaf transection of the destructed palisade and spongy parenchyma cells, showing dead but intact epidermal cells, dead palisade and spongy parenchyma cells, 125 X.

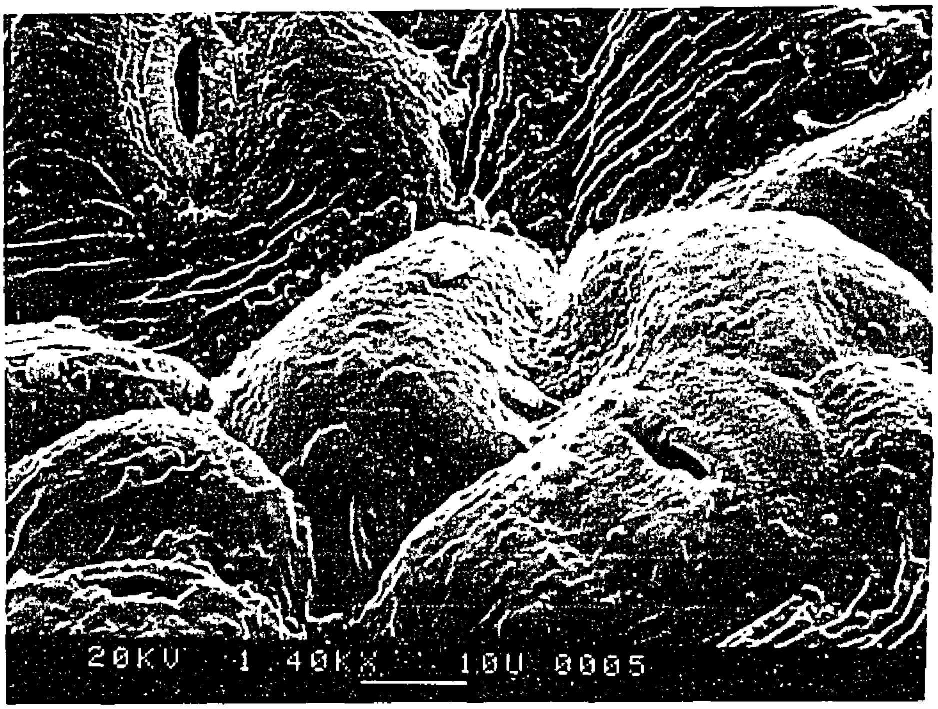


Figure 7. Normal epidermal cells and stomata, showing fully expanded epidermal cells and open stomata, 1,400 X, 20,000 volt, the bar is equal to 10 u.

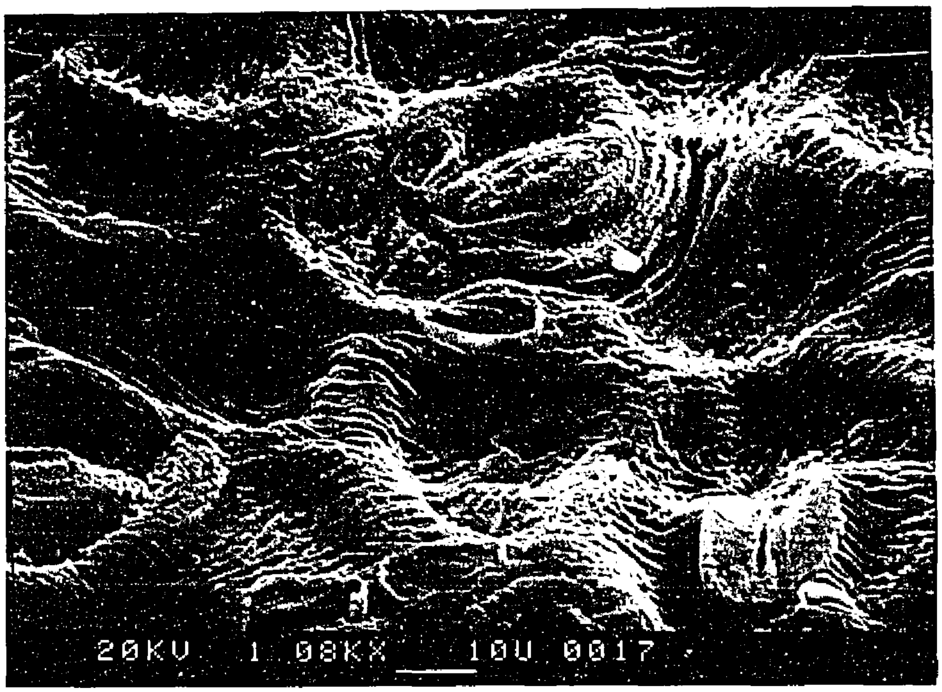


Figure 8. Collapsed epidermal cells and closed stomata of necrotic leaf-spot, 1,080 X, 20,000 volt, the bar is equal to 10 u.


Figure 9. Destructed epidermis of necrotic leaf-spot, showing popped-up epidermis, 720 X, 20,000 volt, the bar is equal to 10 u.

# CONCLUSIONS

The necrotic leaf-spot mutation of cotton is characterized by long term necrotic symptoms, which are sustained throughout the growing season. The symptoms occur in mature leaves. Generally the developmental rate of the necrotic symptoms is very slow and positively related to vegetative growth. The symptoms are quite heterogeneous, and the effect of the mutation on growth can be detected during the late season.

The genetic studies indicated that the necrotic leaf-spot mutation is controlled by a single recessive nuclear gene. The necrotic symptoms appear when cotton plants are homozygous for the mutant alleles. Neither is the mutation associated with male sterile background, nor does it interact with extranuclear gene(s).

The mutation has a small deleterious effect on pollen viability, but not on embryo viability.

The necrotic leaf-spot is cytologically characterized early by the changed staining pattern, then contraction and destruction of palisade and spongy parenchyma cells sequentially. The changed staining pattern and the contraction of the palisade and spongy parenchyma cells are associated with the original dark spot, or later the dark ring around necrotic area. The stomata might be involved, or affected, by the necrosis.

# APPENDIX A

RAW DATA OF THE GENETIC TESTS IN 1989†
(NOR = Normal Plant, NEC = necrotic Plant)

| Lot | Crosses | NOR | NEC | Total |
|---|---|---|---|---|
| | | --- Number --- | | |
| B11 | A6-1-87$F_1$§ (hirs cyto‡ X TM1++)S# | 29 | 5 | 34 |
| B12 | A6-11-87$F_1$ (hirs cyto X TM1)S | 36 | 8 | 44 |
| C1,8 | A6-13-87$F_1$ (hirs cyto X TM1)S | 64 | 14 | 78 |
| C2,3 | A6-18-87$F_1$ (hirs cyto X TM1)S | 56 | 16 | 72 |
| C4 | A6-19-87$F_1$ (hirs cyto X TM1)S | 25 | 8 | 33 |
| C5 | A6-27-87$F_1$ (hirs cyto X TM1)S | 20 | 13 | 33 |
| C6A | A6-3-87$F_1$ (hirs cyto X TM1) X B4-87 hirs cyto | 9 | 5 | 14 |
| C6B | A6-11-87$F_1$ (hirs cyto X TM1) X B4-87 hirs cyto | 6 | 3 | 9 |
| C6C* | A6-17-87$F_1$ (hirs cyto X TM1) X B4-87 hirs cyto | 9 | 4 | 13 |
| C7* | A6-18-87$F_1$ (hirs cyto X TM1) X B4-87 hirs cyto | 16 | 12 | 28 |
| C9 | B4-2,4-87 hirs cyto X A6-87$F_1$ (hirs cyto X TM1) | 25 | 21 | 46 |
| C10 | B4-16,18-87 hirs cyto X A6-87$F_1$ (hirs cyto X TM1) | 15 | 23 | 38 |
| C11* | B1-13-87$F_1$ (TM1 X hirs cyto)S | 38 | 0 | 38 |

| | | | | |
|---|---|---|---|---|
| B1,C12 | B1-14-87$F_1$ (TM1 X hirs cyto)S | 64 | 14 | 78 |
| D2 | B1-17-87$F_1$ (TM1 X hirs cyto)S | 35 | 11 | 46 |
| D3 | B1-18-87$F_1$ (TM1 X hirs cyto)S | 29 | 11 | 40 |
| D4 | B1-21-87$F_1$ (TM1 X hirs cyto)S | 31 | 10 | 41 |
| D5 | B1-23-87$F_1$ (TM1 X hirs cyto)S | 32 | 10 | 42 |
| D6 | B1-27-87$F_1$ (TM1 X hirs cyto)S | 34 | 11 | 45 |
| | | | | |
| D7A | B1-1-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 10 | 9 | 19 |
| D7B | B1-2-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 10 | 6 | 16 |
| D8A | B1-3-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 10 | 14 | 24 |
| D8B | B1-6-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 13 | 6 | 19 |
| D9A | B1-12-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 11 | 9 | 20 |
| D9B* | B1-13-87$F_1$ (TM1 X hirs cyto) X B5-87 hirs cyto | 21 | 1 | 22 |
| J1B,I2A | B1-7-87$F_1$ (TM1 X hirs cyto) X B4-87 hirs cyto | 13 | 13 | 26 |
| I2B | B1-29-87$F_1$ (TM1 X hirs cyto) X B4-87 hirs cyto | 13 | 10 | 23 |
| I2C,J1A | B1-36-87$F_1$ (TM1 X hirs cyto) X B4-87 hirs cyto | 11 | 12 | 23 |
| | | | | |
| D11B | B5-19, 20-87 hirs cyto X B1-7-87F1 (TM1 X hirs cyto) | 5 | 4 | 9 |
| | | | | |
| D12,E1,2* | B2-1-87$F_1$ ($D_{2-2}$ $cyto^{+++}$X TM1)S | 76 | 37 | 113 |
| E3 | B2-3-87$F_1$ ($D_{2-2}$ cyto X TM1)S | 30 | 5 | 35 |
| E5 | B2-7-87$F_1$ ($D_{2-2}$ cyto X TM1)S | 20 | 13 | 33 |
| E6 | B2-25-87$F_1$ ($D_{2-2}$ cyto X TM1)S | 20 | 19 | 39 |

| | | | | |
|---|---|---|---|---|
| E7A | B2-2-87$F_1$ ($D_{2-2}$ cyto X TM1) X B4-87 hirs cyto | 14 | 9 | 23 |
| E7B | B2-25-87$F_1$ ($D_{2-2}$ cyto X TM1) X B4-87 hirs cyto | 11 | 7 | 18 |
| E8A | B2-26-87$F_1$ ($D_{2-2}$ cyto X TM1) X B4-87 hirs cyto | 13 | 13 | 26 |
| E9A,9B | B2-30-87$F_1$ ($D_{2-2}$ cyto X TM1) X B4-87 hirs cyto | 23 | 18 | 41 |
| | | | | |
| E10,11 | B2-1-87$F_1$ ($D_{2-2}$ cyto X TM1) X B6-87 $D_{2-2}$ cyto | 38 | 32 | 70 |
| E12 | B2-44-87$F_1$ ($D_{2-2}$ cyto X TM1) X B6-87 $D_{2-2}$ cyto | 15 | 9 | 24 |
| | | | | |
| E4,L2 | B3-1-87$F_1$ (TM1 X $D_{2-2}$ cyto)S | 248 | 66 | 314 |
| F1A | B3-10-87$F_1$ (TM1 X $D_{2-2}$ cyto)S | 21 | 7 | 28 |
| F1B,2A | B3-15-87$F_1$ (TM1 X $D_{2-2}$ cyto)S | 22 | 14 | 36 |
| F3A | B3-4-87$F_1$ (TM1 X $D_{2-2}$ cyto)S | 22 | 6 | 28 |
| | | | | |
| F5B | B6-8-87 $D_{2-2}$ cyto X B3-87$F_1$ (TM1 X $D_{2-2}$ cyto) | 11 | 6 | 17 |
| F6A,6C | B6-2-87 $D_{2-2}$ cyto X B3-87$F_1$ (TM1 X $D_{2-2}$ cyto) | 13 | 6 | 19 |
| F6B | B6-10-87 $D_{2-2}$ cyto X B3-87$F_1$ (TM1 X $D_{2-2}$ cyto) | 14 | 7 | 21 |
| F7A | B6-9-87 $D_{2-2}$ cyto X B3-87$F_1$ (TM1 X $D_{2-2}$ cyto) | 8 | 4 | 12 |
| F7B | B6-11,12-87 $D_{2-2}$ cyto X B3-87$F_1$ (TM1 X $D_{2-2}$ cyto) | 5 | 9 | 14 |
| | | | | |
| F4A | B3-2-87$F_1$ (TM1 X $D_{2-2}$ cyto) X B4-87 hirs cyto | 5 | 10 | 15 |
| F4B | B3-28-87$F_1$ (TM1 X $D_{2-2}$ cyto) X B4-87 hirs cyto | 4 | 7 | 11 |
| F5A | B3-29-87$F_1$ (TM1 X $D_{2-2}$ cyto) X B4-87 hirs cyto | 8 | 3 | 11 |
| | | | | |
| H1A | C1-3-87 $D_{2-2}$ cyto X B1-87$F_1$ (TM1 X hirs cyto) | 18 | 0 | 18 |

| | | | | | |
|---|---|---|---|---|---|
| H1B,2A | C1-9-87 | $D_{2-2}$ cyto X B1-87$F_1$ (TM1 X hirs cyto) | 20 | 0 | 20 |
| H2B | C1-11-87 | $D_{2-2}$ cyto X B1-87$F_1$ (TM1 X hirs cyto) | 19 | 0 | 19 |
| H2C,I1A | C1-10-87 | $D_{2-2}$ cyto X B1-87$F_1$ (TM1 X hirs cyto) | 27 | 0 | 27 |

† All lines, except TM1, were derived from the necrotic parent-lines AG272 and AG273.

§ $F_1$ = the first filial generation of the cross.

‡ hirs cyto = necrotic-*G. hirsutum* cytoplasm.

++ TM1 = Texas Maker 1.

# S = self-pollination.

* indicates that the crosses are partially contaminated and/or not included in the genetic analysis.

+++ $D_{2-2}$ cyto = necrotic-*G. harknessii* cytoplasm.

# APPENDIX B

## RAW DATA FOR THE POLLEN VIABILITY(%)† IN 1990

| Hybrid | 7-13-90 | | 7-21-90 | | 8-4-90 | | 8-18-90 | |
|---|---|---|---|---|---|---|---|---|
| | ------- | ------- | ------- | ------- | % | ------- | ------- | ------- |
| (B10B-2-89‡ X TM1++)$F_1$§ | 85 | 81 | 83 | 91 | 87 | 92 | 90 | 93 |
| (TM1 X B10B-2-89)$F_1$ | 91 | 86 | 91 | 95 | 91 | 91 | 90 | 91 |
| (B10C-5-89+++ X TM1)$F_1$ | 0 | 0 | 0 | 0 | 14 | 13 | 7 | 7 |
| (TM1 X B10C-5-89) | 86 | 88 | 88 | 91 | 86 | 81 | 90 | 85 |
| Parent (B10B-2-89)S/5# | 72 | 81 | 92 | 89 | 72 | 70 | 93 | 84 |
| Parent (B10C-5-89) S/8 | 0 | 0 | 8 | 7 | 13 | 10 | 8 | 8 |
| Standard TM1 S/44 | 93 | 94 | 95 | 96 | 94 | 93 | 94 | 95 |

(Sampling Date spans the four date columns.)

† The number is the average of duplicated slides.
‡ B10B-2-89 = AG273, necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
§ $F_1$ = the first filial generation of the cross.
+++ B10C-5-89 = AG272, necrotic-*G. harknessii* cytoplasm.
# S/N = the generation (N) of self-pollination.

# APPENDIX C

## RAW DATA FOR THE EMBRYO VIABILITY(%) IN 1990

| Hybrid | Embryo Viability | |
|---|---|---|
| | ------------ % ----------- | |
| (B10B-2-89[‡] X TM1[++])F1[§] | 96 | 92 |
| (TM1 X B10B-2-89)F1 | 94 | 92 |
| (B10C-5-89[+++] X TM1)F1 | 94 | 96 |
| (TM1 X B10C-5-89) | 92 | 94 |
| (B10B-2-89) S/5[#] | 78 | 88 |
| (B10C-5-89) S/8 | 90 | 88 |
| TM1 S/44 | 88 | 90 |

‡ B10B-2-89 = AG273, necrotic-*G. hirsutum* cytoplasm.
++ TM1 = Texas Maker 1.
§ $F_1$ = the first filial generation of the cross.
+++ B10C-5-89 = AG272, necrotic-*G. harknessii* cytoplasm.
\# S/N = the generation (N) of self-pollination.

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