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Allelic Composition of Cotton at the Le_1 and Le_2 Loci.

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ABSTRACT

Cotton (*Gossypium* spp.) zygotes bearing a Le_2^{dav} allelle in combination with Le_1 and/or Le_2 undergo a physiological reaction resulting in embryo or seedling inviability. All F_1 hybrids between cultivated tetraploid cotton (*G. hirsutum* L. and *G. barbadense* L., $2n = 4x = 52$) and a Le_2^{dav} homozygote are inviable. A breeding procedure being developed for mass extraction of doubled haploids of cotton relies on the "bio-elimination" of F_1 hybrids by this complementary lethal system. Success is contingent on the absence of le_1le_2 gametes from a prospective parent because these alleles are fully compatible with Le_2^{dav}. The primary objective of this research was to determine the frequency of le_1 and le_2 in a representative sample of USA-developed *G. hirsutum* cultivars. A secondary objective was to determine if the timing of the incompatibility reaction was consistent across genetic backgrounds. Fifty-two cultivars were tested by observing the frequencies of nonviable vs. viable progeny and the timing of necrosis following two types of matings: (*i*) cultivar $\times$ $le_1le_1Le_2^{dav}Le_2^{dav}$ and (*ii*) (cultivar $\times$ $le_1le_1le_2le_2$) $\times$ $le_1le_1Le_2^{dav}Le_2^{dav}$. All 52 cultivars were found to be $Le_1Le_1Le_2Le_2$, indicating that the frequencies of alleles le_1 and le_2 are zero, or nearly so, in USA-developed *G. hirsutum* germplasm. Results on necrotic development indicated that the cumulative dosage of alleles Le_1 and Le_2 affected the onset of necrosis in the presence of Le_2^{dav}. Because alleles le_1 and le_2 are rare in American upland cottons, the doubled-haploid breeding system will be applicable to these stocks.

METHODS THAT EXPEDITE inbreeding and inbred development are advantageous to plant breeders and geneticists. Doubled-haploid (DH) extraction constitutes the most extreme form of inbreeding and the most rapid method of pure-line development. Where paternal haploids can be extracted via agamospermy, DH recovery also facilitates cytoplasmic-nuclear substitutions (Kermicle, 1969). Many procedures for DH extraction have been reported (Kasha, 1974), but application often has been hindered by high cost and special requirements of time and facilities.

The semigamy mutant from Pima cotton (*G. barbadense* L.), discovered by Turcotte and Feaster (1963), has been a useful tool for cotton genomic manipulations, including extraction of modest numbers of inbreds and cytoplasmic substitutions (Turcotte and Feaster, 1969, 1973, 1982; Mahill et al., 1983, 1984). Stelly et al. (1988) have proposed combining the semigamy trait with a component of the D_3 complementary-lethal system, introgressed from *G. davidsonii* Kell. (Lee, 1981a,b), to form a hybrid-eliminating, haploid-producing (HEHP) system to be used with colchicine treatment of seeds for mass extraction of doubled-haploids of cotton. Female parents to be used for HEHP extraction will be homozygous for the semigamy allele, *Se*, and the lethal factor, Le_2^{dav}. Application of the HEHP system requires that the paternal plants, from which DH are to be derived, be homozygous for Le_1 and/or Le_2, if true hybrids are to be "bio-eliminated" by the incompatibility system (Table 1). Efficacy of the HEHP system for recurrent selection in complex populations will require that all parents used to form the population be homozygous for Le_1 and Le_2; otherwise, le_1le_2 gametes will arise from segregation, compromising the lethal system's ability to eliminate all nonagamospermic progeny. HEHP extraction of DH from a biparental F_1, or a generation derived from it, would require that both

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Table 1. Phenotypes of *Gossypium hirsutum* and *G. barbadense* L. according to genotypes at the Le_1 and Le_2 loci.†

Genotype		Phenotype
– –	$le_2\ le_2$	Viable
– –	$Le_2\ le_2$	Viable
$le_1\ le_1$	$Le_2\ Le_2$	Viable‡
$le_1\ le_1$	$le_2\ Le_2^{dav}$	Viable
$le_1\ le_1$	$Le_2^{dav}\ Le_2^{dav}$	Viable
Le_1 –	– Le_2^{dav}	Inviable
– –	$Le_2\ Le_2^{dav}$	Inviable

† Alleles at Le_1 locus include le_1 and Le_1; alleles at Le_2 locus include le_2, Le_2, and Le_2^{dav} (Lee, 1981a).
‡ All USA cultivars currently tested carry this genotype.

parents be homozygous for at least Le_1 or Le_2, but not necessarily both. The frequencies of le_1 and le_2 are therefore of considerable practical importance to the HEHP breeding procedure. The Le_2^{dav} allele has been incorporated into a few glandless cotton (*G. hirsutum* L.) lines to preclude contamination from outcrossing with the glanded types, as proposed by Lee (1981b). The frequencies of the recessive compatibility alleles are similarly important to the use of Le_2^{dav} as an isolation mechanism.

Specific objectives of this research were to determine: (i) the frequency of le_1 and le_2 in a representative sample of USA *G. hirsutum* L. cultivars, and (ii) if the time of embryo and/or seedling death depends on the dosage of Le_1 and/or Le_2 and possibly other factors.

MATERIALS AND METHODS

Two experiments were conducted to determine cultivar genotypes at the Le_1 and Le_2 loci, and to detect if dosage of Le_1 and Le_2 influenced the time of seedling lethality. Each experiment consisted of (*i*) a series of cross pollinations followed by (*ii*) a screening of seedlings to determine the presence and timing of the lethality reaction. A set of 52 cultivars originating over time from numerous breeding programs across the USA was used to represent the overall USA *G. hirsutum* germplasm. The set of 52 cultivars were provided by J.C. McCarty, Jr. from the cultivar component of the Stoneville, MS, Cotton Germplasm Collection. The cultivars were assumed to be true-breeding pure lines for the Le_1 and Le_2 loci.

Five seedlings of each cultivar were mechanically transplanted into the cotton cytogenetics research field in College Station, TX. Forty *G. hirsutum* seedlings with the genotype $le_1le_1le_2le_2$ and 40 *G. hirsutum* tester stock seedlings of the genotype $le_1le_1Le_2^{dav}Le_2^{dav}$, descended from seed provided by J.A. Lee, were transplanted into the same field.

Experiment 1

To determine which cultivars, if any, were $le_1le_1le_2le_2$, each was crossed as female to $le_1le_1Le_2^{dav}Le_2^{dav}$ during the summer of 1986. All pedigrees were maintained by plant and seeds were stored at room temperature.

In February 1987, up to 30 F_1 seeds from each testcross were planted in peat pellets in a heated greenhouse (ca. 26°C). Seedlings were watered well and treated every 3 d with fungicide for control of common cotton seedling diseases. Emergence dates for seedlings were marked daily with colored-coded toothpicks. Ten days after planting, all nonemerged seed were exhumed and examined as to possible causes for their nonemergence. The number of days after emergence to seedling necrosis was recorded. Emergence was defined as that time when the hypocotyl broke through the soil surface. Daily ratings for the lethal reaction were made for each seedling relative to maternal line and emergence date.

Experiment 2

To determine which cultivars, if any, were monomeric, i.e., $Le_1Le_1le_2le_2$ or $le_1le_1Le_2Le_2$, each cultivar was crossed initially with $le_1le_1le_2le_2$ and the derived F_1 was crossed with $le_1le_1Le_2^{dav}Le_2^{dav}$. Initial crosses were made onto field-grown plants used as female during the summer of 1986 and then onto greenhouse-grown F_1 plants as female in the winter of 1986 to 1987. Seed from the latter crosses were used for the seedling screening tests in March 1987. For 10 cultivars, inadequate numbers of testcross seed [$F_1 \times le_1le_1Le_2^{dav}Le_2^{dav}$] were available to conduct a statistically significant determination among their respective genotypes; therefore, additional testcrosses were made in the field during the summer of 1987 and the resulting testcross seed were similarly screened during the fall of 1987. To distinguish between the first screening and those tested later, we herein designated them as Groups A and B, respectively.

Preliminary tests indicated germination to be erratic; so, the seed to be screened were chemically delinted with concentrated sulfuric acid, well-rinsed, and then physically scarified because of a hard seed coat factor present in the paternal parent, $le_1le_1le_2le_2$ (not used in Exp. 1). Seed were classified daily for germination as defined by emergence of the radicle from the seed coat. The seed coat was removed on the day of germination so that symptoms of the lethal reaction could be rated prior to seedling growth. The seedling trial subsequently followed the same format as in Exp. 1, with similar subjective definitions for emergence and necrosis. Viable plants were those showing no symptoms of the incompatibility reaction after 21 d.

Data were entered on DBASE II and transferred via ASCII files to SYSTAT for graphic and univariate statistical analyses. Chi-square values were calculated without the Yates correction factor.

RESULTS AND DISCUSSION

In Exp. 1, crosses between cultivars and $le_1le_1Le_2^{dav}Le_2^{dav}$ yielded seed that produced three types of phenotypic response; (*i*) no germination; (*ii*) germination, but seedling death prior to emergence; and

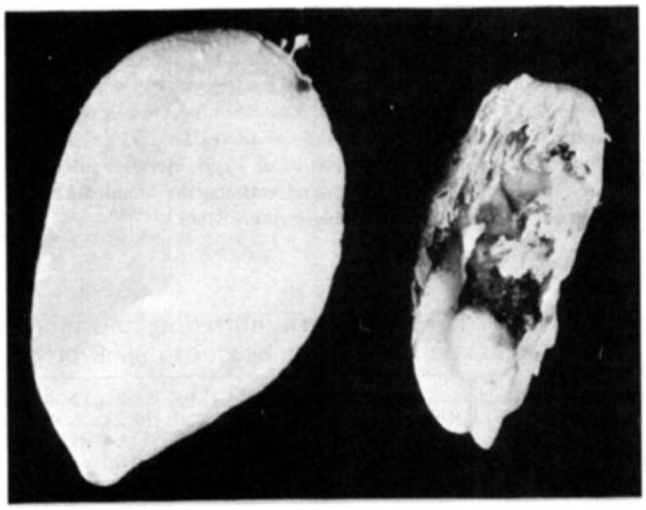

Fig. 1. Comparison of mature embryos of cotton (*Gossypium hirsutum* L.) Left: normal embryo. Right: necrotic embryo with symptoms characteristic of the Le_2^{dav} lethal reaction.

(*iii*) emergence followed by seedling death. No family produced viable progeny, indicating that no cultivar tested had the $le_1le_1le_2le_2$ genotype. Only 45% of the planted seed emerged and all seedlings became necrotic within a period of 7 d after emergence.

Exhumation revealed that 42.0% (273/650) of the nonemergent seed germinated, but died prior to emergence. Closer inspection revealed symptoms characteristic of the lethal reaction. Darkened necrotic tissue was scattered through out the young cotyledons and contrasted sharply with the light yellow healthy tissue (Fig. 1). Interestingly, the radicle and other developing roots were apparently unaffected by the reaction. The remaining nonemergent seed (58.0% or 377/650) rotted without evidence of germination.

The time after emergence until death ranged from 1 to 7 d, with family means ranging from 0.0 (no seedlings emerged) to 5.8 d. The cumulative mean calculated from all 52 families was 3.9 d, with a standard deviation of 1.4 d (Fig. 2). All seed either failed to emerge or died before 8 d, indicating that all cultivars were monomeric and homozygous for at least Le_1 or Le_2.

In Exp. 2, segregation ratios among testcross progenies were used to classify genotypes of individual cultivars at the Le_1 and Le_2 loci. Progenies from (cultivar $\times$ $le_1le_1le_2le_2$) $\times$ $le_1le_1Le_2^{dav}Le_2^{dav}$ were expected to segregate 3:1, 1:1, or 0:1 (inviable:viable), depending on cultivar genotype. In no testcross family were all progeny viable (Table 2), confirming results from Exp. 1. Observed total segregation for each family was compatible with the ratio of 3:1 (Table 2) and no family had a segregation pattern compatible with a 1:1 ratio. These results indicated that all 52 cultivars are dimeric and homozygous for the dominant alleles, i.e., $Le_1Le_1Le_2Le_2$.

Chi-square tests for pooled data in Group A revealed that segregation (1114:336) fit the results expected for digenic segregation P(3:1) > 0.20 (χ^2_{1df} = 2.58), but not that for monogenic segregation P(1:1) < 0.01 (χ^2_{1df} = 417.44) (Table 2). However, when the results for Group A and B were pooled, the segregation was 1440:411. Due to an accentuated deficiency of viable types, the observed segregation no longer fit the results expected for digenic segregation P(3:1) < 0.05 (χ^2_{1df} = 7.72). However, the departure from a 1:1 ratio, expected under monogenic segregation, was much larger, P(1:1) < 0.01 (χ^2_{1df} = 573.04) (Table 2). The departure from a 3:1 ratio can be attributed to extraneous factors that lessened viability of some $le_1le_1le_2Le_2^{dav}$ seed. Given the large sample size, small changes in viability, e.g. 5%, would yield statistically significant departures from a 3:1 ratio. Overall levels of heterogeneity among families were negligible within Groups A, B, and A and B combined.

Frequency of emergence was higher in Exp. 2 than in Exp. 1 (89% vs. 45%), due in part to differences in planting procedure between the two experiments. In Exp. 2, more than 60% of the seedlings that did not emerge exhibited symptoms of the lethal reaction at germination. This suggested that the lethal reaction precluded emergence of many seedlings, an inference supported by the observation that the percentage of seedlings perishing between germination and emergence was much higher for germinated seed displaying symptoms (310/412, 75%) than for those lacking symptoms (10/1048, 1%).

In Exp. 2, the number of days between emergence and death of inviable seedlings for family means ranged from 4.0 to 9.2 d with a mean of 6.4 and a standard deviation of 1.5 d. There was little correlation (r = 0.12) in post-emergence days-to-death among related families screened in Exp. 1 vs. Exp. 2 Group A. This may have arisen due to differences between the two

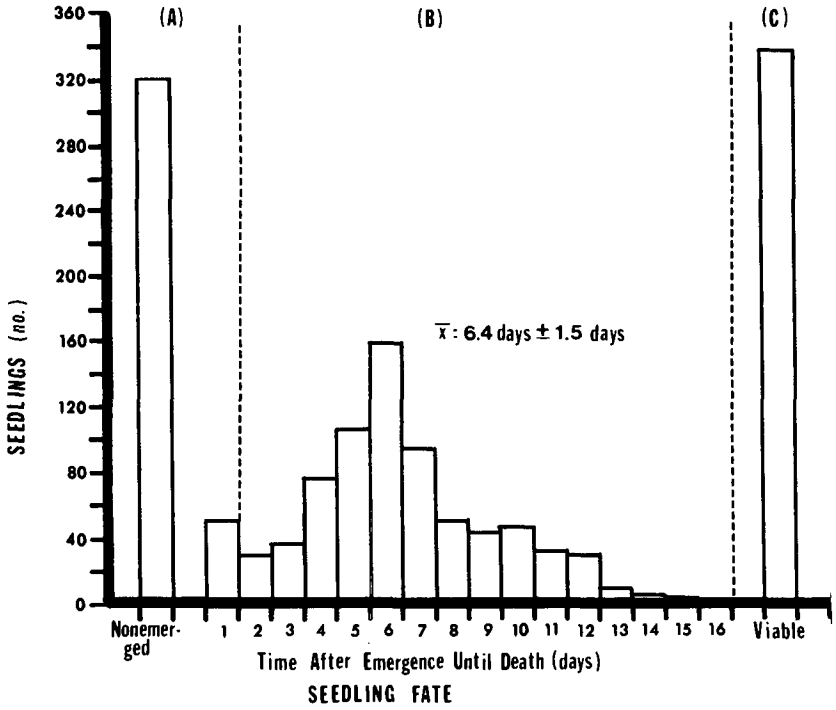

Fig. 2. Histogram of cotton seedling fate in Exp. 1 for pooled data over 52 cultivars ($Le_1Le_1Le_2Le_2$) crossed with $le_1le_1Le_2^{dav}Le_2^{dav}$.

Table 2. Segregation within 52 testcross progenies, i.e., (cultivar × $le_1le_1le_2le_2$) × $le_1le_1Le_2^{dav}Le_2^{dav}$ for viability vs. inviability in *Gossypium hirsutum* L..

Cultivar	S.A. No.†	Seedlings		Chi-square values			
		Lethal	Viable	3:1		1:1	
Acala Nunn's 5-37	234	16	5	0.02		5.76*‡	
Acala 1064 (NM)	238	24	9	0.09		6.82*	
Coker's Cleveland	248	23	7	0.04		8.53*	
Coker's Wilds 2	265	18	8	0.46		3.85*	
Lone Star	282	10	2	0.44		5.33*	
Wannamakers Early Wilt	297	26	5	1.30		14.23*	
Stoneville 2B Original	308	26	7	0.25		10.94*	
Acala 5675	335	36	6	2.57		21.43*	
Pandora	347	26	6	0.67		12.50*	
Deltatype Webber	395	20	7	0.01		6.26*	
Deltapine 12	459	25	7	0.17		10.12*	
Delta Smooth Leaf	477	26	8	0.04		9.53*	
Acala 111, Rogers	517	30	6	1.33		16.00*	
Half + Half	864	24	9	0.09		6.82*	
Stoneville 213	1163	23	7	0.04		8.53*	
Carolina Queen	1165	26	5	1.30		14.23*	
Bayou B-10	1174	26	8	0.04		9.53*	
Acala SJ-1	1181	25	6	0.53		11.65*	
Coker 201	1182	32	4	3.70		21.78*	
Deltapine 45A	1187	26	8	0.04		9.53*	
Dixie King	951	24	6	0.40		10.80*	
Kasch	890	26	8	0.04		9.53*	
Missdel	881	25	8	0.01		8.76*	
Coker 124	954	23	7	0.04		8.53*	
Hi-Bred	962	26	6	0.67		12.50*	
Plains	965	26	8	0.04		9.53*	
Stardel	970	22	5	0.61		10.70*	
Wacona 8	1007	19	8	0.31		4.48*	
Blightmaster	1020	19	6	0.01		6.76*	
Paymaster 101	1021	28	6	0.98		14.24*	
Coker 100A	1056	15	4	0.16		6.37*	
Gregg 35	1123	24	6	0.40		10.80*	
Lankart Sel. 611	1141	25	8	0.01		8.76*	
Delfos 9169	1151	25	8	0.01		8.76*	
Deltapine 15	1152	16	6	0.06		4.55*	
Empire WR-61	1158	23	8	0.01		7.26*	
Fox 4	1159	23	5	0.76		11.57*	
Delcot 277	1193	31	8	0.42		13.56*	
Lockett 4789A	1196	29	5	1.92		16.94*	
Coker 5110	1214	17	6	0.01		5.26*	
Acala 1517-70	1225	26	8	0.04		9.53*	
Stoneville 731N (98731)	1333	17	6	0.01		5.26*	
Deltapine 16	1186	37(15)§	17(5)	1.21	(0.00)	7.41*	(5.00*)
Deltapine 14	874	53(32)	20(9)	0.22	(0.20)	14.92*	(12.90*)
Cleveland 54	246	43(38)	12(11)	0.30	(0.17)	17.47*	(14.88*)
Coker 100	252	54(46)	16(12)	0.17	(0.57)	20.63*	(19.93*)
Lockett 140-46	509	55(41)	11(4)	2.45	(6.23*)	29.33*	(30.42*)
Mebane Watson	516	42(30)	13(7)	0.06	(0.73)	15.29*	(14.30*)
Lankart Sel. 57	1131	44(38)	12(11)	0.38	(0.17)	18.29*	(14.88*)
Pope	996	43(21)	15(3)	0.02	(2.00)	13.52*	(13.50*)
Wilds 15	474	31(24)	8(2)	0.42	(4.15*)	13.56*	(18.62*)
Rowden 41B Bryant	522	41(41)	11(11)	0.41	(0.41)	17.31*	(17.31*)
TOTAL (Group A)		1114	336	29.73		439.33	
TOTAL (Group B)		326	75	14.63		161.74	
			Group A				
Sum of individual X^2 (51 df)				29.73		439.33	
X^2 of totals (1 df)				2.58		417.44**	
Homogeneity (50 df)				27.15		21.89	
			Group B				
Sum of individual X^2 (10 df)				14.63		161.74	
X^2 of totals (1 df)				8.48**		157.11**	
Homogeneity (9 df)				6.15		4.63	
			Groups A and B				
Sum of individual X^2 (52 df)				25.69		585.55	
X^2 of totals (1 df)				7.72**		573.04**	
Homogeneity (51 df)				17.97		12.51	

* $P(X^2_{1df} > 3.84) < 0.05$.
** $P(X^2_{1df} > 6.64) < 0.01$.
† Stoneville acession number.
‡ A nonsignificant difference from a 3:1 ratio and a significant difference from a 1:1 indicated that a cultivar was of the $Le_1Le_1Le_2Le_2$ genotype.
§ All numbers not in parentheses are Groups A (early screening) and B (later screening) combined. Numbers in parentheses are from Group B (later screening) only.

experiments in seed preparation. Equally plausible explanations include interactions of the lethality system with enviromental conditions, i.e., temperature-sensitivity of the lethal reaction (Phillips, 1977), and interractions involving genetic modifiers (Lee, 1981a).

Lee (1981a) stated that seed of genotype $Le_1le_1Le_2Le_2^{dav}$ became necrotic earlier than $Le_1le_1le_2Le_2^{dav}$ and $le_1le_1Le_2Le_2^{dav}$. He also inferred that earliness and intensity of the incompatibility reaction was different for $Le_1le_1le_2Le_2^{dav}$ and $le_1le_1Le_2Le_2^{dav}$, although he could not determine which had a stronger effect. Thus, the relative order of intensity hypothesized was $Le_1le_1Le_2Le_2^{dav} > (Le_1le_1le_2Le_2^{dav} > le_2le_2Le_2Le_2^{dav} >$, or vice-versa) $le_1le_1le_2Le_2^{dav}$ where only the last genotype is viable. All seedlings in Exp. 1 progeny tests were of the genotype $Le_1le_1Le_2Le_2^{dav}$. According to Lee (1981a), seed with this genotype becomes necrotic from midembryonic to very early seedling stages. The cumulative average for Exp. 1 was 3.9 d after emergence (seed that died prior to emergence were not included in the average). In Exp. 2, seed screened in the progeny test were segregating 1:1:1:1 for $Le_1le_1Le_2Le_2^{dav}$: $Le_1le_1le_2Le_2^{dav}$: $le_1le_1Le_2Le_2^{dav}$: $le_1le_1le_2Le_2^{dav}$. Depending on the magnitude of difference among Le_1Le_2-Le_2^{dav}, Le_1-Le_2^{dav}, and Le_2-Le_2^{dav} interactions relative to genetic background, the Exp. 2 progeny were expected to show phenotypic ratios for time of death 1:1:1:1, 1:2:1, or 3:1. The trimodality of graphed data suggested a 1:2:1 segregation ratio (Fig. 3), which most logically corresponds to (A) $Le_1le_1Le_2Le_2^{dav}$, (B) $Le_1le_1le_2Le_2^{dav}$ and $le_1le_1Le_2Le_2^{dav}$, and (C) $le_1le_1le_2Le_2^{dav}$. Continuity of the distribution indicated that phenotypic overlap occurred. Experiment 1 and results of Lee (1981a) showed

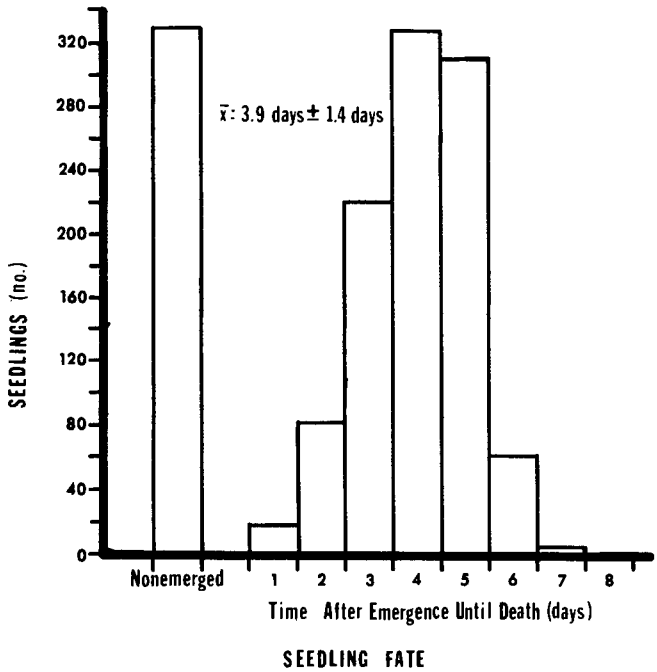

Fig. 3. Histogram of cotton seedling fate in Exp. 2 for pooled data over 52 cultivars $Le_1Le_1Le_2Le_2$ testcrossed as (cultivars × $le_1le_1le_2le_2$) × $le_1le_1Le_2^{dav}Le_2^{dav}$. Proposed genotypes for classes are (A) $Le_1le_1Le_2Le_2^{dav}$, (B) $Le_1le_1le_2Le_2^{dav}$ and $le_1le_1Le_2Le_2^{dav}$, and (C) $le_1le_1le_2Le_2^{dav}$.

that some $Le_1le_1Le_2Le_2^{dav}$ seedlings emerge, indicating that emergence vs. nonemergence is not itself a sufficient criterion to identify $Le_1le_1Le_2Le_2^{dav}$ genotypes. In Exp. 2, more seedlings died at Day 1 (58) than at Day 2 (30) and 3 (36), suggesting that $Le_1le_1Le_2Le_2^{dav}$ contributed to the total number of deaths on Day 1. From the distribution of Fig. 2, especially the minimum frequency observed at Day 2, we inferred that classes A and B could be roughly ascribed to death before Day 2 vs. on Day 2 or later. According to this classification system, χ^2 test results were P(1:2:1) > .50, (χ^2_{2df} = 2.12). The lack of a bimodal 1:1 distribution within class B (Fig. 2) for Exp. 2 data (pooled or within Groups) indicates that Le_1-Le_2^{dav} and Le_2-Le_2^{dav} interactions were similar in intensity, were obscured by other sources of variation, or both. Large scale testing within families would be required to demonstrate the tetramodal distribution predicted by Lee's model.

In Exp. 1, all progeny were $Le_1le_1Le_2Le_2^{dav}$ and should have died by the first day after emergence, based on observations from Exp. 2. The onset of lethality, however, occurred up to 7 d postemergence. A report by Phillips and Reid (1975) indicated that the lethal reaction was suppressed if the seed developed at temperatures of greater than 37°C during the day. This may account for the relative delay of necrosis for $Le_1le_1Le_2Le_2^{dav}$ seedlings in Exp. 1 vs. Exp. 2 because Exp. 2 seed was formed in a greenhouse during the winter where temperatures never approached 37°C. At the lower temperatures, necrosis could have begun during embryogenesis in Exp. 2, rather than during germination as presumably happened in Exp. 1.

To identify the specific control of necrosis initiation, it will be necessary to use more rigorous experimental controls than were employed here. It will be necessary to produce four *G. hirsutum* isolines with genotypes $Le_1Le_1Le_2Le_2$, $Le_1Le_1le_2le_2$, $le_1le_1Le_2Le_2$, and $le_1le_1le_2le_2$ and to use environments as uniform as possible for each stage of experimentation. Nevertheless, the results obtained clearly confirm Lee's digenic model in a larger sample of *G. hirsutum*, and conform to Lee's inference that $Le_1le_1Le_2Le_2^{dav}$ dies before $Le_1le_1le_2Le_2^{dav}$ and $le_1le_1Le_2Le_2^{dav}$. However, they also indicate the onset of necrosis is affected by additional parameters. Most importantly, the results demonstrate that the frequencies of alleles le_1 and le_2 are very low in USA *G. hirsutum* cultivars, and suggest that the HEHP system proposed for DH extraction will be generally applicable to USA *G. hirsutum* germplasm.

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