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Inheritance of necrotic mutants of groundnut (*Arachis hypogaea* L.)

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Abstract

In groundnut, parent Dharwad Early Runner on treatment with ethyl methane sulphonate and 5-azacytidine (a demethylating agent) resulted in two independent lesion mimic mutants namely, Necrotic 1 and Necrotic 2 belonging to Spanish (var. *vulgaris*) and Valencia (var. *fastigiata*) type, respectively. Inheritance studies involving Non-necrotic 1, Non-necrotic 2 (Non-necrotic versions of Necrotic 1 and Necrotic 2) and parent Dharwad Early Runner as ovule parents and Necrotic 1 and Necrotic 2 as pollen parents indicated that necrosis phenotype is under the control of five genes. Though the two necrotic mutants were isolated independently and belongs to different botanical groups, they are essentially similar with respect to necrotic phenotype and genotype. But, Non-necrotic 1, Non-necrotic 2 and parent Dharwad Early Runner differ for their allelic constitution in two or three genes. Response to 5-azacytidine (a demethylating agent) suggested that Dharwad Early Runner is in hypermethylated state, necrotic mutants *viz*., Necrotic 1 and Necrotic 2 are in moderately-methylated state, while non-necrotic variants and Dharwad Early Runner like variants are in hypomethylated state. Results indicate the possible 'epigenetic' nature of these necrotic mutants.

Key words: Necrotic mutants, peanut, epigenetic

Introduction

Disease lesion mimics are a class of mutants that promote the production of discrete leaf lesion in the absence of obvious stress, injury or disease on the plants and resemble some known pathological condition. Lesion mimics have been reported in plants as early as 1923 [1]. Since then, both spontaneous and induced lesion mimic mutants have been reported in several plant species like tomato (*Lycopersicon esculentum*), maize (*Zea mays*), barley (*Hordeum vulgare*), *Arabidopsis thaliana*, soybean (*Glycine max*), rice (*Oryza sativa*), wheat (*Triticum aestivum*) and groundnut (*Arachis hypogaea*) [2-4].

In our groundnut breeding programme, 'Dharwad Early Runner' (DER) sharing the characters of the two subspecies (ssp. *hypogaea* and ssp. *fastigiata*) on treatment with ethyl methane sulphonate (EMS), gamma-rays and 5-azacytidine (a demethylating agent) resulted in a very high frequency of mutants belonging to all the four botanical types *viz*., Virginia bunch, Virginia runner (var. *hypogaea*), Valencia (var. *fastigiata*) and Spanish bunch (var. *vulgaris*) [5-7]. DER on treatment with EMS (0.2%) and 5-azacytidine (5mM) resulted in two independent lesion mimic mutants namely, 'Necrotic 1' and 'Necrotic 2' belonging to Spanish and Valencia type, respectively (Figs. 1B, 1E). The development of necrosis in both the mutants starts as inter-veinal yellowing of leaves followed by necrotic disease like symptoms (reddening / light brown) and collapsing of necrotic regions in the advanced stage (Figs. 1C, 1F). The symptoms initially start in the fourth leaf from bottom of at the seedling stage of the plant and appear in all the newly emerging leaves in the absence of any stress or pathogen. However, the initial three leaves remain non-necrotic throughout the life of the plant indicating the developmental regulation of necrosis [8]. When examined, the lesions were not found associated with any pathogens like bacteria (by ooze test), fungi (by culturing on potato dextrose agar medium) and virus (by electron microscopy). As per the classification of Dietrich *et al*. [9], these mutants are determinate type with slow and restricted expansion of lesions.

The mutants bred true over generations but occasionally produced non-necrotic versions that resembled mutants in gross morphology but without any

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necrosis (Figs. 1A, 1D) and also revertants completely resembling the original DER parent (Fig. 1H). Two necrotic mutants, their non-necrotic versions *viz*. Non-necrotic 1 and Non-necrotic 2 and the parent DER were utilized to determine the inheritance of necrosis. One of the mutants (Necrotic 2) was a product of treatment with 5-azacytidine, a demethylating agent (Fig. 1D) and both the necrotic mutants had all the features of epimutants like the occurrence of reversions and new variants [10]. To gain further insight into this nature, the mutants and their non-necrotic versions were assessed for their response to 5-azacytidine.

Materials and methods

Using 'Non-necrotic 1', 'Non-necrotic 2' and 'DER' as ovule parents and 'Necrotic 1' and 'Necrotic 2' as pollen parents, six crosses were made during the rainy season of 2001. The hybrid seeds of the cross Non-necrotic 2 x Necrotic 1 could not be recovered due to the high incidence of stem and pod rot. The other five crosses were advanced from F_1 through F_3 generations during post-rainy 2001, rainy 2002 and post-rainy 2002 seasons as plant to progeny rows and each pedigreed plant were assessed for necrosis. The segregation for non-necrotic and necrotic phenotypes was ascertained in each cross. The chi-square test was employed to fit the F_2 and F_3 data to the expected ratios as per the genetic hypotheses.

Hundred seeds each of Necrotic 1, Necrotic 2, Non-necrotic 1 and Non-necrotic 2 were soaked in water for 12 hours and then treated with 5-azacytidine (10mM) for 1 hour. Treated seeds were thoroughly washed in running tap water and sown immediately to grow A_1 generation during the rainy season of 2001. They were advanced from A_1 to A_2 and A_3 generations during post-rainy 2001, rainy 2002 and post-rainy 2002 seasons as plant progeny rows and studied for their behaviour with respect to necrosis and growth habit.

Results and discussion

Inheritance of necrotic mutants

In the cross, Non-necrotic 1 x Necrotic 1, the F_1 was 'necrotic' and segregation in the F_2 generation showed a good fit to the ratio of 15 non-necrotic: 241 necrotic (Table 1). Families in the F_3 generation also behaved as expected based on the F_2 ratio. Segregation in the F_2 and F_3 generations suggests that 'necrotic' phenotype is under 'tetragenic' control with two duplicate dominant basic genes (A, B) essential for 'non-necrotic' phenotype and two duplicate inhibitory genes (C, D) which inhibit the expression of A and B genes leading to 'necrotic' phenotype.

In the cross, Non-necrotic 2 x Necrotic 2, the F_1 was 'non-necrotic' and segregation in the F_2 generation showed a good fit to the ratio of 45 non-necrotic: 19 necrotic (Table 1). In the F_3 generation, slightly higher chi-square values were observed for the expected behaviour of families, which could be due to small population size. Segregation in the F_2 and F_3 generations suggests that 'necrotic' phenotype is under 'trigenic' control i.e. two complementary duplicate genes (A, B) leading to 'non-necrotic' phenotype and one anti-inhibitory gene (E) which suppresses the two inhibitory genes (C, D) present in both 'Non-necrotic 2' and 'Necrotic 2'.

In the crosses, DER x Necrotic 1 and DER x Necrotic 2, the F_1 was 'non-necrotic' and segregation in the F_2 generation showed a good fit to the ratio of 735 non-necrotic: 289 necrotic (Table 1). Behaviour of families in the F_3 generation was as expected. Segregation in the F_2 and F_3 generations indicate that 'necrotic' phenotype is under the control of 'five' genes *i.e*. two duplicate dominant basic genes (A, B) essential for 'non-necrotic' phenotype and two duplicate inhibitory genes (C, D) which inhibit the expression of A and B leading to 'necrotic' phenotype and one anti-inhibitory gene (E) which suppress the two inhibitory genes (C, D) leading to 'non-necrotic' phenotype. Similar behaviour in both the crosses with the parent DER revealed the same genetic make up for lesion development in both the mutants.

The cross Non-necrotic 1 x Necrotic 2 also showed a similar F_2 ratio of 15 non-necrotic: 241 necrotic as in the cross Non-necrotic 1 x Necrotic 1 (Table 1). Behaviour of families in the F_3 generation was as expected. Thus again confirming the similar genetic constitution of both the mutants for lesion development.

On considering all the five crosses, the 'necrotic' phenotype seems to be under the control of 'five' genes i.e. two duplicate dominant basic genes (A, B) essential for 'non-necrotic' phenotype and two duplicate inhibitory genes (C, D) leading to 'necrotic' phenotype and one anti-inhibitory gene (E) which suppress the two inhibitory genes (C, D) leading to 'non-necrotic' phenotype. The results indicate that though the two necrotic mutants are independently isolated and belong to different botanical groups they are essentially similar with respect to 'necrotic' phenotype and genotype. Based on the results, the genotypic constitution of Necrotic 1

Table 1. Segregation of necrotic and non-necrotic types in F_2 and F_3 generations of five crosses involving necrotic mutants, non-necrotics and Dharwad Early Runner (DER)

Cross	Generation	No. of progeny	Phenotype		Expected ratio (Non-necrotic : Necrotic)	Chi-square	df	Probability
			Non-necrotic	Necrotic				
Non-necrotic 1 x	F_2	1	4	80	15:241	0.183	1	0.50-0.70
Necrotic 1	F_3	42	-	594	BT[a] (Necrotic)	-	-	-
		10	35	116	1:3	0.267	1	0.50-0.70
		10	16	222	1:15	0.089	1	0.70-0.90
		5	24	115	15:49	2.951	1	0.05-0.10
		5	11	69	3:13	1.328	1	0.20-0.30
		4	5	140	3:61	0.499	1	0.30-0.50
		4	5	104	15:241	0.321	1	0.50-0.70
		1	1	-	BT (Non-necrotic)	-	-	-
		2	30	9	3:1	0.077	1	0.70-0.90
		1	41	2	15:1	0.189	1	0.50-0.70
Total		84	-	-	119:30:28:16: 16:16:16: 7:4:4	2.087	9	0.95-1.00
Non-necrotic 2 x	F_2	6	88	29	45:19	1.345	1	0.20-0.30
Necrotic 2	F_3	25	427	-	BT (Non-necrotic)	-	-	-
		27	469	112	3:1	10.149	1	0.001-0.01
		15	323	24	15:1	0.262	1	0.50-0.70
		9	105	71	9:7	0.831	1	0.30-0.50
		12	201	89	45:19	0.139	1	0.70-0.90
		30	-	491	BT (Necrotic)	-	-	-
Total		118	-	-	7:18:4:8:8:19	23.856**	5	<0.001
Non-necrotic 1 x	F_2	2	3	113	15:241	2.256	1	0.10-0.20
Necrotic 2	F_3	69	-	1359	BT (Necrotic)	-	-	86
		165	1:3	11.486	1	<0.001		
		12	25	304	1:15	1.023	1	0.30-0.50
		4	20	80	15:49	0.659	1	0.30-0.50
		6	20	109	3:13	0.766	1	0.30-0.50
		5	11	192	15:241	0.071	1	0.70-0.90
		2	14	-	BT (Non-necrotic)	-	-	-
		0	-	-	3:1	-	-	-
		1	33	3	15:1	0.267	1	0.50-0.70
Total		114	-	-	119:30:28:16: 16:16: 16:7:4:4	10.148	8	0.20-0.30
DER x Necrotic 1	F_2	5	76	28	735:289	0.087	1	0.70-0.90
	F_3	20	370	-	BT (non-necrotic)	-	-	-
		17	149	48	3:1	0.042	1	0.70-0.90
		9	182	12	15:1	0.001	1	0.95-1.00
		5	129	26	13:3	0.397	1	0.50-0.70
		5	44	20	45:19	0.075	1	0.70-0.90
		5	48	12	49:15	0.394	1	0.50-0.70
		4	37	33	9:7	0.329	1	0.50-0.70

Table 1 contd.....

Cross	Generation	No. of progeny	Phenotype		Expected ratio (Non-necrotic : Necrotic)	Chi-square	df	Probability
			Non-necrotic	Necrotic				
		3	23	12	39:25	0.335	1	0.50-0.70
		2	17	11	147:109	0.124	1	0.70-0.90
		3	33	9	195:61	0.134	1	0.70-0.90
		3	48	19	735:289	0.0006	1	0.95-1.00
		16	-	123	BT (necrotic)	-	-	-
		3	6	20	1:3	0.051	1	0.70-0.90
		3	4	57	1:15	0.010	1	0.90-0.95
		2	4	19	3:13	0.027	1	0.70-0.90
		2	8	34	15:49	0.449	1	0.50-0.70
		1	1	17	15:241	0.004	1	0.95-1.00
		1	1	22	3:61	0.006	1	0.90-0.95
Total		104	-	-	133:174:76:56:56:56:56:32:32:32:32:169:28:28:16:16:16:16	5.386	17	0.70-0.90
DER x Necrotic 2	F_2	1	29	10	735:289	0.129	1	0.70-0.90
	F_3	5	105	-	BT (non-necrotic)	-	-	-
	F_3	6	115	35	3:1	0.222	1	0.50-0.70
		4	110	7	15:1	0.014	1	0.90-0.95
		3	63	12	13:3	0.371	1	0.50-0.70
		2	50	22	45:19	0.026	1	0.70-0.90
		3	59	15	49:15	0.412	1	0.500-0.70
		1	4	3	9:7	0.002	1	0.95-1.00
		1	4	2	39:25	0.081	1	0.70-0.90
		1	7	5	147:109	0.004	1	0.95-1.00
		2	69	16	195:61	1.171	1	0.20-0.30
		1	5	3	735:289	0.338	1	0.50-0.70
		5	-	88	BT (heterotic)	-	-	-
		1	3	8	1:3	0.009	1	0.90-0.95
		1	1	14	1:15	0.004	1	0.95-1.00
		1	2	6	3:13	0.010	1	0.90-0.95
		0	-	-	15:49	-	-	-
		1	2	11	15:241	0.098	1	0.70-0.90
		1	1	21	3:61	0.0009	1	0.95-0.100
Total		39	-	-	133:174:76:56:56:56:56:32:32:32:32:169:28:28:16:16:16:16	4.110	17	0.95-1.00

[a] BT – Breeding True; **Significant at 0.001 probability

(aabbCCDDee), Necrotic 2 (aabbCCDDee), Non-necrotic 1 (AABBccddee), Non-necrotic 2 (AABBCCDDEE) and DER (AABBccddEE) are deduced. The necrotic mutants are genotypically same though they are of independent origin but the two non-necrotics and DER differ for allelic constitution in two or three genes.

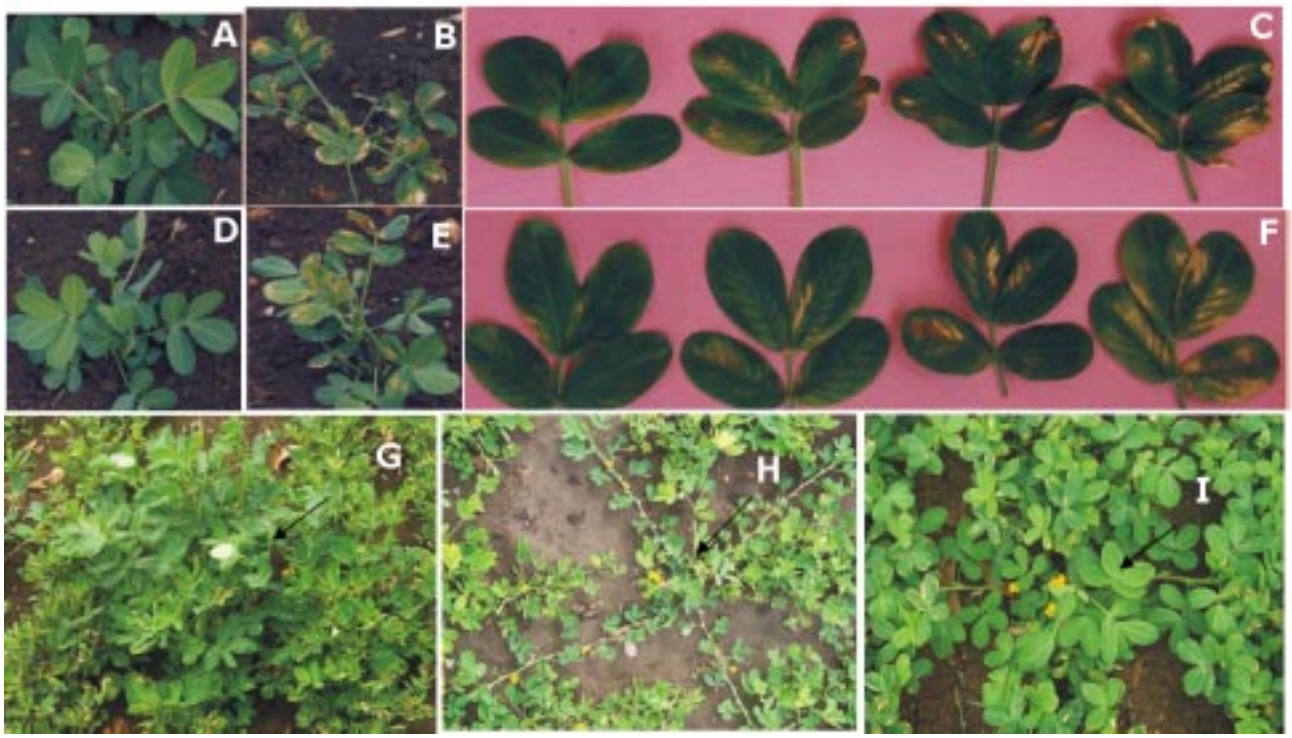

Fig. 1. Non-necrotics, Necrotic mutants, developmental stages of necrosis and Non-necrotic variants, DER like variants and DER like revertants: (A) Non-necrotic 1 derived from Necrotic 1 mutant (B) Necrotic 1 mutant (C) Developmental stages of necrosis in Necrotic 1 mutant (D) Non-necrotic 2 derived from Necrotic 2 mutant (E) Necrotic 2 mutant (F) Developmental stages of necrosis in Necrotic 2 mutant (G) Non-necrotic variants (V_1) in Necrotic progeny rows (H) DER like revertant in Necrotic progeny rows (I) DER like variants (V_2) in Necrotic progeny rows

Earlier, rusty leaf trait in groundnut was reported to be under the control of two complementary recessive genes [3] or suppressor genes [4]. In soybean, the disease lesion mimic showing chlorotic and/or necrotic lesions on the leaves was shown to be under the control of a recessive nuclear allele [11], while three necrotic root mutants were shown to be governed by a recessive gene and were allelic [12]. A novel mutation in arabidopsis, *hlm1*, which causes lesion mimic phenotype, segregated as single recessive allele [13]. Lesion mimics are reported to be mostly under the control of one or two genes but in the present study necrosis is found to be under the control of five genes. Mutants affecting several traits in groundnut have been reported to be under the control of several genes [14, 15] as observed in the present study which is probably due to the allotetraploid nature of groundnut.

Response to 5-azacytidine

To gain further insight, the mutants and their non-necrotic versions were assessed for their response to 5-azacytidine. On 5-azacytidine treatment of 100 seeds of Necrotic 1, 63 plants were raised in the A_1 generation. In the A_2 generation, 36-bred true (57.14%) for necrotic phenotype, while other 27 segregated (42.86%) for necrotics and non-necrotic variants (V_1: resemble mutants in gross morphology except for necrosis phenotype; Fig. 1G) ($1.85x10^{-2}$ to $25x10^{-2}$: V_1). The non-necrotic variants (V_1) were observed with a frequency of 5.06 per cent in the segregating families. When they were assessed for their behaviour in A_3 generation, out of 45 non-necrotic variants, 24 bred true (53.33%) for non-necrotic phenotype, while 21 segregated (46.67%) for non-necrotic and necrotic plants with varying frequencies. DER like variants (V_2: spreading growth habit like DER but not true revertants; Fig. 1I) were not observed in segregating necrotic lines of A_2 and A_3 generations.

On 5-azacytidine treatment of 100 seeds of Necrotic 2 mutant, 67 plants were raised in the A_1 generation. In the A_2 generation, 51 bred true (76.12%) for necrotic phenotype, while 16 lines segregated (23.88%) for non-necrotic variants (V_1; Figure 1G) and

DER like variants (V_2; Figure 1I) ($1.02x10^{-2}$ to $20x10^{-2}$: V_1 and V_2). The non-necrotic variants were observed with a frequency of 2.77 per cent in the segregating necrotic families. Out of the 16 lines, eight lines (11.94%) segregated for necrotic plants and non-necrotic variants (V_1), seven lines (10.45%) segregated for necrotic plants and DER like variants (V_2), while only one line (1.49%) segregated for necrotic plants, non-necrotic variants (V_1) and DER like variants (V_2). All the 17 non-necrotic variants *viz.* non-necrotic variants (V_1) (5) and DER like variants (V_2) (12) were studied as progeny rows in A_3 generation. Out of five non-necrotic variants (V_1), three-bred true (60%) for non-necrotic phenotype, while other two segregated for non-necrotic and necrotic plants. Among the 12 DER like variants (V_2), only two bred true (16.67%), while other 10 segregated (83.33%) for non-necrotic and necrotic plants.

Necrotic 1 and Necrotic 2 on treatment with 5-azacytidine resulted in non-necrotic variants (V_1) and/or DER like variants (V_2) but did not result in DER like revertants (Fig. 1H). Non-necrotic 1 and Non-necrotic 2 were also treated with 5-azacytidine but they did not show any change either to the necrotic type or DER like variants/revertants.

These results suggest that parent DER is in 'hypermethylated' state, while mutants Necrotic 1 and Necrotic 2 are in 'moderately-methylated' state. On treatment of these mutants with 5-azacytidine (a demethylaing agent) they result in 'hypomethylated' non-necrotic variants (V_1) and DER like variants (V_2) but not DER like revertants (hypermethylated). This view is also supported by the lack of response of non-necrotic versions to 5-azacytidine treatment. Further, occasional occurrence of revertants resembling DER and non-necrotic variants among the necrotic mutants (Fig. 1H, 1G) suggest the 'epigenetic' nature of these mutants [10] with simultaneous change in several genes. Polyploids are known to respond epigenetically to 5-azacytidine [16, 17] and alkylating agents like EMS [18].

Impaired activities of uroporphyrinogen decarboxylase (UROD) in maize and protopophyrinogen oxidase (PPO) in Arabidopsis, the key enzymes in the biosynthetic pathway of chlorophyll and heme in plants, have been shown to be resulting in disease lesion mimic phenotype [19, 20]. Thus, a similar impairment might have occurred in these mutants leading to 'necrotic' phenotype. Earlier, molecular analysis of lesion mimic mutants has led to identification of certain factors and/or regulators such as *LSD1* gene which encodes a putative zinc finger protein [21], *dnd1* and *cpn1* which encode calcium related proteins; CNGC-2, a cation channel that can conduct calcium; a copine a calcium-dependent phospholipids binding protein [22, 23] and *hlm*1 which encode a member of the CNGC (Cyclic nucleotide-gated channel) ion channel family [13]. Hence, these mutants are the better candidates which could be used in future to unearth the biochemical and molecular basis of 'necrotic' phenotype as they come from the same genetic background.

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