To the Graduate Council:

I am submitting herewith a thesis written by Hendratno entitled "Induced Variability of Resistance to Root-Knot Nematodes (*Meloidogyne incognita*) in Soybeans (*Glycine max*)." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Agricultural Biology.

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INDUCED VARIABILITY OF RESISTANCE TO ROOT-KNOT NEMATODES (*MELOIDOGYNE INCOGNITA*) IN SOYBEANS (*GLYCINE MAX*)

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

The purpose of this study was to determine the variability of resistance to root-knot nematodes (*Meloidogyne incognita*) in soybeans (*Glycine max*) induced by seed treatment with ethylmethane sulfonate (EMS).

Two soybean varieties, 'Essex' and 'Forrest,' were chosen as the experimental materials mainly on the basis of their distinct level of resistance; 'Essex' is susceptible and 'Forrest' is resistant. The M_2 and control populations were grown and tested for nematode resistance in the greenhouse. The number of galls developed on the roots was used to indicate the level of resistance.

The frequency distribution of the M_2 population of 'Essex' was found to be significantly different from that of the control ($P < 0.01$). The variability of resistance was broadened by the EMS treatment. The M_2 population of 'Forrest' showed no significant difference in the frequency distribution as compared with the control ($0.50 < P < 0.60$). The EMS treatment did not significantly alter the variability of resistance.

It is suggested that the probability of altering the variability of resistance to root-knot nematodes through induced mutations is greater in susceptible varieties than in resistant varieties.

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CHAPTER I

INTRODUCTION

Adequate genetic variability is an essential factor in plant improvement and the induction of mutations is one of the ways to increase variability. The potential of the induction of mutations in plant improvement is attested by the increasing number of crop varieties developed through induced mutations. A world-wide summary recorded 98 crop varieties and 47 ornamentals (Sigurbjornsson and Micke, 1974). Of the 98 crop varieties, 91 were released in the period between 1957 and 1973 and 62 of these 91 were released within the last six years before 1973. All of the 47 ornamentals were released within the period of 1962-1973. Fifty-five percent of the 98 crop varieties are cereals, 21 percent are legumes and the rest are fruit trees and other crops. With respect to soybeans four mutant varieties have been released, two in the Republic of China and the other two in Japan.

The type of improvement obtained through induced mutations involved disease resistance as well as many other characters. In fact, 24 of the released varieties developed through induced mutations have increased resistance to certain diseases. One of the remarkable accomplishments was the development of 'Todd's Mitcham' peppermint (*Mentha piperita* L.) which is highly resistant to *Verticillium* sp. (Murray and Todd, 1972; 1975). This *Verticillium* wilt-resistant peppermint was successfully developed through induced mutations without changing the oil quality and yield.

This thesis is particularly concerned with root-knot nematode resistance in soybeans. An assessment of the crop losses caused by plant parasitic nematodes in the United States revealed that root-knot nematodes contribute approximately 4 percent yield loss in soybeans (Good, 1968). The use of resistant varieties is the most economical and effective method of controlling root-knot nematodes.

Very little information is available on the induction of mutations in plants with respect to nematode resistance. Numerous agents are available for inducing mutations. These include physical mutagens (X-rays, gamma rays, neutrons) and chemical mutagens. There is no convincing evidence which shows the superiority of either group to the other with respect to the spectrum of mutations induced. The use of the chemical mutagen, ethylmethane sulfonate (EMS), in the present study was not based on any assumption that chemical mutagens are superior. EMS has been used extensively in higher plants and as a member of the alkylating agents the mechanism by which it causes mutations has been studied in detail (Freese, 1971).

The aim of the present study was to obtain information on the induction of mutations with EMS in two varieties of soybean with respect to the changes of the level of resistance to root-knot nematodes [Meloidogyne incognita (Kofoid and White) Chitwood]. The experiment was conducted in the greenhouse of the Agricultural Biology Department of the University of Tennessee in the Winter and Spring 1976.

CHAPTER II

LITERATURE REVIEW

I. CONCEPTS AND CRITERIA OF RESISTANCE

Wingard (1953), in the 1953 Yearbook of Agriculture, defined resistance as the ability of a plant to withstand, oppose, lessen, or overcome the attack of a pathogen. Nematologists, however, tend to place emphasis on the development of nematode populations. In general, a resistant plant is one on which nematodes reproduce poorly (Rohde, 1972). Dropkin (1955) points out that there are two separate factors in resistance, one is the suitability of the host for the life of the parasite and the other is the effect of the parasite on the well-being of the host. The relation between these two factors and the resistance was summarized by Dropkin and Nelson (1960) as follows:

		Host Growth	
		Good	Poor
Parasite Growth	Good	tolerant	susceptible
	Poor	resistant	intolerant

In this description the distinction was made between resistance and tolerance. A plant which is infested by a high number of actively reproducing nematodes but which shows little indication of injury is considered to be tolerant.

It is clear that resistance to nematodes can be measured by the reaction of the plant to the parasite as well as the effect of the plant

on the reproduction of the nematodes. Both criteria are important but the former is of more practical use than the latter.

Plant reaction or response to root-knot nematodes is divided into two major categories (Bird, 1974): (a) responses of the whole intact plant, and (b) cellular responses. The first consists of morphological and physiological responses and the second refers to hyperplasia and the formation of giant cells. The most obvious and observable morphological response of the whole intact plant is the formation of characteristic galls on the roots.

II. THE NATURE OF RESISTANCE TO ROOT-KNOT NEMATODES

Numerous reports are available on the relationship between root-knot nematodes and their hosts. According to Goplen and Stanford (1959) the early concept of resistance of plants to root-knot nematodes was based on the failure of larvae to enter the roots. Barrons (1939), however, obtained results which showed that resistance was not related to the nematode penetration. In his studies he found that the larvae freely entered the roots of both resistant and susceptible plants.

Christie (1946; 1949) found no correlation between the susceptibility of the host and the extent of invasion of roots by root-knot nematode larvae. He observed that the susceptibility was related to the subsequent development and reproduction of the nematodes inside the roots.

Liao and Dunlap (1950) obtained results that were in favor to the early concept. They observed that the larvae of *Meloidogyne* sp. freely penetrated the roots of susceptible *Lycopersicon esculentum* but only a few larvae entered the roots of resistant *Lycopersicon peruvianum*.

Sasser and Taylor (1952) made an attempt to clarify the problem. They extended the work of Barrons (1939) and Christie (1946; 1949) and finally came to the conclusion that resistance is not of the same nature in all plants but depends on the plant species and the nematode species involved. Three cases may be observed: (a) failure of the larvae to enter the roots, (b) entry of reduced numbers of larvae with little or no development, and (c) entry of large numbers of larvae with varying degrees of development ranging from none to a few of the individuals reaching maturity.

Goplen and Stanford (1959) reported a total failure of penetration of Meloidogyne hapla larvae into the roots of resistant alfalfa varieties. In resistant soybean varieties, Dropkin and Nelson (1960) observed a decreased penetration of Meloidogyne hapla larvae.

In most cases the nematodes invade both resistant and susceptible plants without showing any notable difference in the number of nematodes penetrating the roots. In the resistant plants, however, the number of nematodes after invasion decreases rapidly as the result of the hypersensitive reactions of the hosts where necrotic cells are formed and wall off the invading larvae to prevent the development of the larvae. The mechanism of this phenomenon is not fully understood. In some plants the hypersensitive reaction has been attributed to the rapid accumulation of chlorogenic acid (Pi and Rohde, 1967; Milne, Boshoff, and Buchan, 1965).

The absence or incomplete development of giant cells is also an important factor in resistance because giant cells are necessary for the development of adult females. The histopathological studies by Dropkin

and Nelson (1960) on 19 soybean varieties indicated that the rapid nematode growth and abundant egg production in the susceptible varieties were associated with the complete development of giant cells.

III. THE INHERITANCE OF RESISTANCE TO ROOT-KNOT NEMATODES

According to Hare (1965), the earliest study on the inheritance of resistance of plants to root-knot nematodes was conducted by Orton in 1911. In this study a resistant variety of cowpea ('Iron') was crossed with a susceptible variety. The F_1 plants were uniformly resistant and a great variation was observed in the F_2. Later Mackie (1934) confirmed that resistance in 'Iron' cowpea was dominant. A number of reports related to the inheritance of resistance to root-knot nematodes in several other plant species are also available (Clayton and Foster, 1940; Barrons, 1940; Smith, 1941; McFarlene et al., 1946; Watts, 1947; Frazier and Dennett, 1949). However, these early studies were based on the wrong taxonomy in which root-knot nematodes were thought to represent a single species.

Chitwood (1949) revised the genus Meloidogyne and made a separation into species and subspecies. This revision provides a firm basis for the study of the inheritance of resistance. Hare (1956; 1957) reported an experimental proof of a single dominant gene for resistance to root-knot nematodes in pepper (Capsicum frutescens). Pepper plants with this gene were resistant to Meloidogyne incognita, M. javanica and M. arenaria but susceptible to M. hapla. A single dominant gene for resistance in tobacco was reported by Drolsom et al. (1958) and Clayton et al. (1958). It was

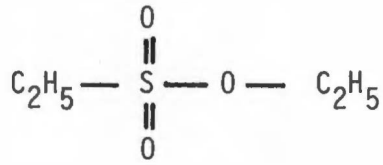
shown by Drolsom and Moore (1958) that lines with this gene were resistant to Meloidogyne incognita but susceptible to M. javanica, M. arenaria and M. hapla. Gilbert and McGuire (1956) described resistance to severe galling by Meloidogyne incognita on tomato as being controlled by a single dominant gene. Barham and Winstead (1957) and Thomason and Smith (1957) stated that the resistance was to Meloidogyne incognita, M. javanica and M. arenaria but not M. hapla.

In some plants the resistance to root-knot nematodes are associated with recessive genes. Smith (1954) found that resistance of Gossypium barbadense var. darwinii to Meloidogyne incognita was recessive and possibly polygenic. In common bean (Phaseolus vulgaris) the resistance to Meloidogyne incognita was found to be controlled by two recessive genes (Blazey et al., 1964). No information is available on the inheritance of resistance to root-knot nematodes in soybeans.

IV. ETHYLMETHANE SULFONATE

Ethylmethane sulfonate (EMS) belongs to a group of chemical mutagens known as alkylating agents. Most, and probably all, alkylating agents have some mutagenic effects which have been shown in many genetic systems such as maize, Vicia, Drosophila, Neurospora, bacteria and phages (Freese, 1971). The mechanisms by which they cause mutations have been extensively studied (Fishbein et al., 1970; Freese, 1971). The alkylating agents can induce point mutations (mainly transitions) and chromosome mutations.

The chemical structure of EMS is shown below:



The initial reaction of this chemical with the genetic material or DNA is the addition of the ethyl group (ethylation) to the DNA bases or to the phosphate groups of nucleic acids. The ethylated base might cause base pairing mistakes during DNA duplication or it might be removed from the DNA and a wrong base incorporated to fill in the gap during the DNA duplication. Ethylation of the phosphate group might ultimately lead to the broken DNA backbone.

CHAPTER III

MATERIALS AND METHODS

I. SOYBEAN VARIETIES

'Essex' and 'Forrest' varieties were chosen as the experimental materials on the basis of the distinctive degree of resistance to root-knot nematodes. 'Essex' is susceptible and 'Forrest' is resistant. In addition both are outstanding varieties in the maturity group V.

'Essex' originated from the cross 'Lee' X S5-7075 (Smith and Camper, 1973). S5-7075 is a selection from the cross N48-1248 X 'Perry.' N48-1248 is a selection from the cross 'Roanoke' X ('Ogden' X 'CNS'). 'Essex' is susceptible to root-knot nematodes, however, nothing is stated in the reference about the resistance or susceptibility of the parentage to root-knot nematodes. With respect to other diseases 'Essex' is resistant to bacterial pustule, several races of downy mildew, and frogeye leafspot, and moderately resistant to phytophthora rot.

'Forrest' originated from the cross 'Dyer' X 'Bragg' (Hartwig and Epps, 1973). In addition to its resistance to root-knot nematodes, 'Forrest' is also resistant to races 1 and 3 of the soybean cyst nematodes, bacterial pustule, wildfire, target spot, and moderatley resistant to phytophthora rot. The source of resistance to root-knot nematodes is traceable back to 'Palmetto,' an introduction variety from China. The resistance has been transferred to 'Forrest' through 'Jackson' and then 'Bragg.'

II. EMS TREATMENT

Ethylmethane sulfonate (EMS) was used as the mutagenic agent. The treatment of the seeds with the EMS followed the procedure recommended by Constantin et al. (1976). Some precautions were taken during the handling of the EMS and the treatment of the seeds. The preparation of the EMS solution and the treatment of the seeds were carefully done in the fume hood to prevent the inhalation of the toxic vapor. The direct contact of the chemical with the skin was avoided by using rubber gloves.

The seeds were presoaked 16 hours in air-bubbled water at room temperature (about 22 C). They were then aerobically soaked in 0.050 M phosphate-buffered solution of EMS (pH : 7.0) for eight hours and finally rinsed thoroughly in running tap water.

III. PLANTING AND SAMPLING PROCEDURES

The seeds were planted wet in 6 m rows at the Plant Science Farm of the University of Tennessee. The seeds were hand-dropped into open furrows (2-3 cm depth) at the rate of about 30 seeds per meter of row. Since the seeds were planted wet it was necessary that the soil at the time of planting was moist. The planted furrows were immediately covered with soil to prevent desiccation of the seeds.

Two hundred eighty plants (ten per row) were sampled at random from both the control and the treated populations of each variety. At the harvesting stage, one three-seeded pod was randomly picked from each sampled M_1 plant.

IV. ROOT-KNOT NEMATODE INOCULATION PROCEDURE

The eggs used as inoculum in this experiment were obtained from a culture of root-knot nematodes (Meloidogyne incognita) in susceptible tomato plants ('Rutgers'). The eggs were extracted from the galled tomato roots of 50 day-old culture.

One hundred pods were randomly taken from each treated lot and fifty pods from each control lot that had been harvested from the M_1 and control populations. One seed was randomly shelled from each pod. The seeds were planted each in a 6 cm pot containing sterile soil-sand mixture (1 : 1). Twelve days after planting each seedling was transplanted into a 13 cm pot. Inoculation was done at the time of transplanting at a rate of about 6,000 eggs per plant. A portion of the inoculum was applied to the surface of the soil-root mass of the seedling and the other portion to the surface of the area on which the seedling was going to be transplanted.

V. DISEASE EVALUATION

The disease evaluation was made 52-60 days after inoculation. The pots were soaked in water for several minutes to facilitate the removal of the plants from the pots and the separation of the roots from the soil particles. After being separated from the tops the roots were carefully washed in running tap water and then observed under a magnifier (1.5 magnification). The number of galls on the roots of each plant were recorded. Only root swellings which were clearly and definitely identifiable as galls were included. The number of galls was used as the criterion to determine the level of resistance of a particular individual

plant. The individuals were classified on the basis of their number of galls according to the standard classification commonly used by nematologists to evaluate the degree of resistance to root-knot nematodes. For 'Essex' variety, in addition to the standard classification, a different classification was also used.

VI. STATISTICAL ANALYSIS

The frequency data were arranged in a contingency table. The chi-square frequency test was used to determine if the frequency distribution of the M_2 population was significantly different from that of the control.

The chi-square frequency test requires two conditions for its validity, first that the observed frequencies are mutually independent, and secondly that frequencies are not too small (Campbell, 1974, p. 98; Remington and Schork, 1970, p. 231). In this particular study, prior knowledge of the populations and random sampling assured the existence of the first condition. With respect to the second condition it is generally suggested that the smallest expected frequency is at least 1 (Snedecor and Cochran, 1972, p. 235). To ensure this condition several adjacent classes were combined.

CHAPTER IV

RESULTS AND DISCUSSION

Frequency distributions illustrated in the figures are expressed as the percentage of the total number of plants observed in each treatment. The number of plants tested in the control and M_2 population of 'Essex' were 40 and 95 respectively. For 'Forrest' the number was 41 and 92.

Fig. 1 illustrates the distribution of the control and M_2 population of 'Essex' based on the following standard classification.

Class	Number of galls per plant
0	0
1	1-2
2	3-10
3	11-30
4	31-100
5	>100

In the control, 95 percent of the plants tested fell into the highest class, i.e. 5. The rest fell into class 4. This clearly indicates the low level of resistance of 'Essex' to root-knot nematodes. The M_2 population showed a different distribution. In this population the highest class was occupied by only 43 percent of the tested individuals. The larger proportion occupied the lower classes; 2 percent in class 2, 11 percent in class 3, and 44 percent in class 4. The frequency distribution of the M_2 population was found to be statistically different ($P < 0.01$) from that of the control as indicated by the chi-square test applied to the data arranged in a contingency table (Table 1). This

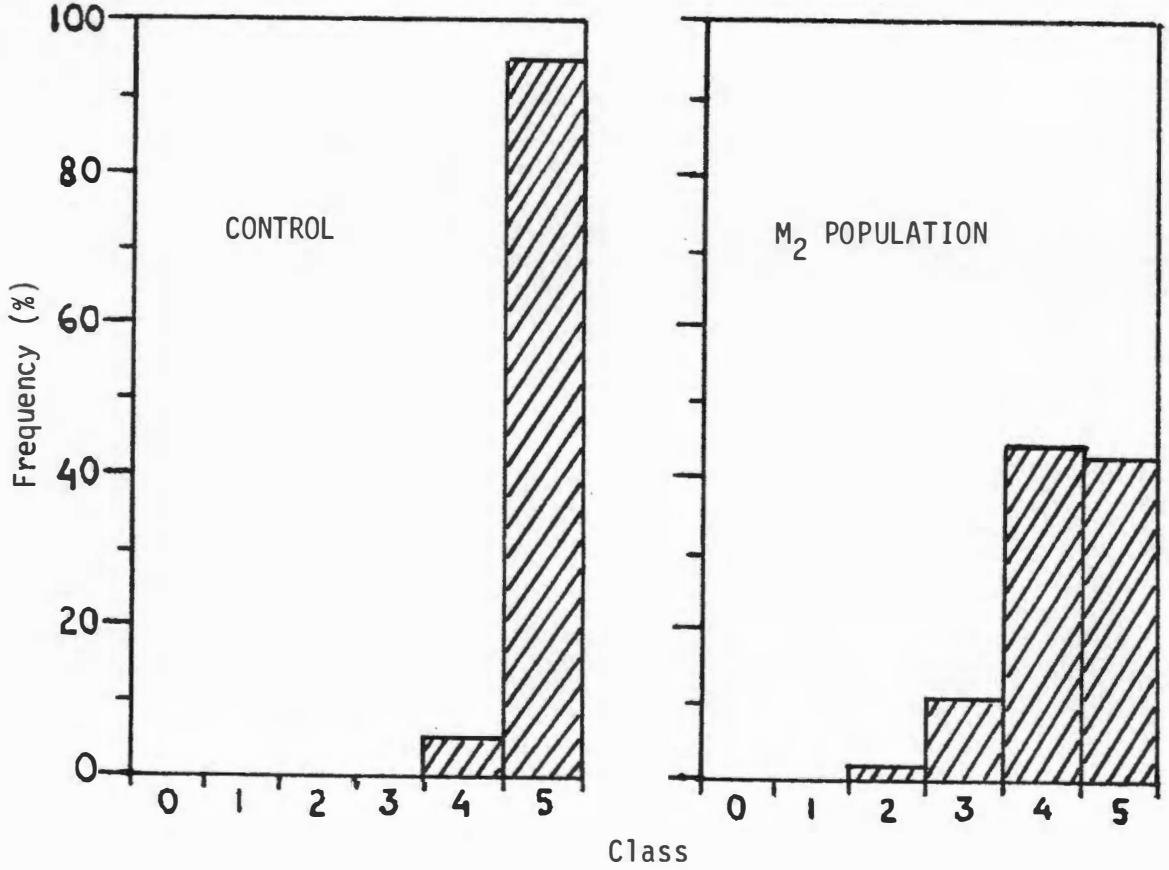


Fig. 1. Frequency distribution of the control and M_2 population of 'Essex' variety, based on the standard classification.

TABLE 1

CONTINGENCY TABLE OF THE CONTROL AND M_2 POPULATIONS OF 'ESSEX' VARIETY BASED ON THE STANDARD CLASSIFICATION

Class*	No. of Galls Per Plant	Observed Frequency: Control Population	Observed Frequency: M_2 Population	Total
0	0	0	0	0
1	1-2	0	0	0
2	3-10	0	2	2
3	11-30	0	10	10
4	31-100	2	42	44
5	>100	38	41	79
Total		40	95	135

*The first four classes were combined to meet the condition for the validity of the chi-square frequency test that the smallest expected frequency should be at least 1.

Chi-square = 31.27 ($P < 0.01$)

means that the variability of the level of resistance was significantly broadened by the EMS treatment. It is assumed that mutations bringing about the changes in the level of resistance to root-knot nematodes were induced. For 'Essex' variety, the results suggest that there is a good probability to select individual plants with a higher level of resistance to root-knot nematodes from the M_2 generation of an EMS-treated population.

According to the standard classification all plants with more than 100 galls are put into the highest class, i.e. 5. Since 'Essex' is susceptible, almost all plants in the control population were in class 5. With the standard classification it is not possible to show the shift of the distribution to the right in the M_2 population, as compared with the control. A different classification which can be used to show the shift of the distribution in either direction would be more appropriate. It is justifiable to use the different classification to obtain a more obvious picture of the distribution shift. The frequency distribution presented in Fig. 2 was set up on the basis of the following classification:

Class	Number of galls per plant
0	0-50
1	51-101
2	102-152
3	153-203
4	204-254
5	255-305
6	>305

Data of this distribution were arranged in a contingency table (Table 2) for the chi-square test. It was found that the frequency distribution

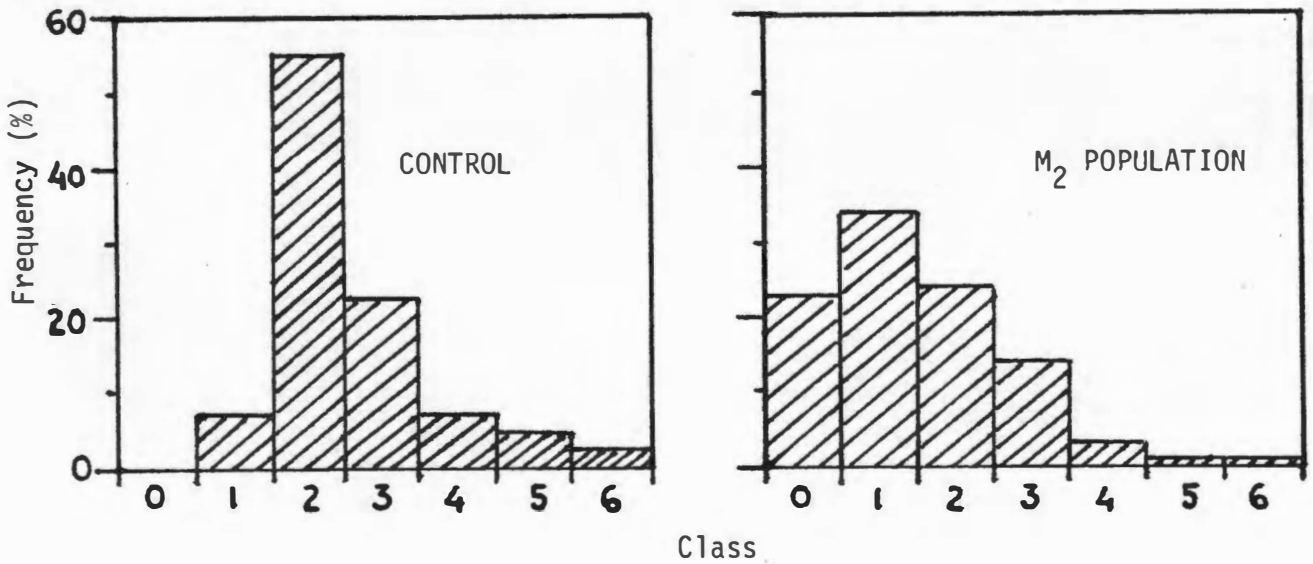


Fig. 2. Frequency distribution of the control and M_2 population of 'Essex' variety, based on the alternate classification.

TABLE 2

CONTINGENCY TABLE OF THE CONTROL AND M_2 POPULATIONS OF 'ESSEX' VARIETY BASED ON THE ALTERNATE CLASSIFICATION

Class*	No. of Galls per Plant	Observed Frequency: Control Population	Observed Frequency: M_2 Population	Total
0	0-50	0	22	22
1	51-101	3	32	35
2	102-152	22	23	45
3	153-203	9	13	22
4	204-254	3	3	6
5	255-305	2	1	3
6	>305	1	1	2
Total		40	95	135

*The three last classes were combined to meet the condition for the validity of the chi-square frequency test that the smallest expected frequency should be at least 1.

Chi-square = 29.36 ($P < 0.01$).

of the M-2 population was statistically different from that of the control. However, it is obviously shown in Fig. 2 that the difference was attributable to the changes in the frequencies of individuals within classes 2, 1, and 0. It was apparent that the distribution shifted to the left and not to the right. This fact emphasizes the previous conclusion that improvement of the resistance level of 'Essex' to root-knot nematodes could be obtained through induced mutations with EMS.

With respect to the 'Forrest' variety, a different situation was observed. The frequency distributions based on the standard classification are presented in Fig. 3. It is clearly shown that the majority of the individuals in the control population (about 98 percent) fall into class 2 or lower. This fact confirms the description of 'Forrest' variety reported by Hartwig and Epps (1973) that it has a very high level of resistance to root-knot nematodes. The frequency distribution was not altered in the M-2 population. The chi-square test applied to the frequency distributions arranged in a contingency table (Table 3) indicated that the two distributions (control and M_2 populations) were not different ($0.50 < P < 0.60$). Since 'Forrest' has already a high level of resistance, there is no point to being concerned about selecting the more resistant individuals. On the contrary, concern about losing the resistance or decreasing the level of resistance is rational and important. The results of this portion of the experiment suggest that the probability of losing or decreasing the resistance of 'Forrest' to root-knot nematodes through EMS-induced mutations is quite low.

Very limited information is available on the induced mutations in plants with respect to the resistance to root-knot nematodes. Offut and

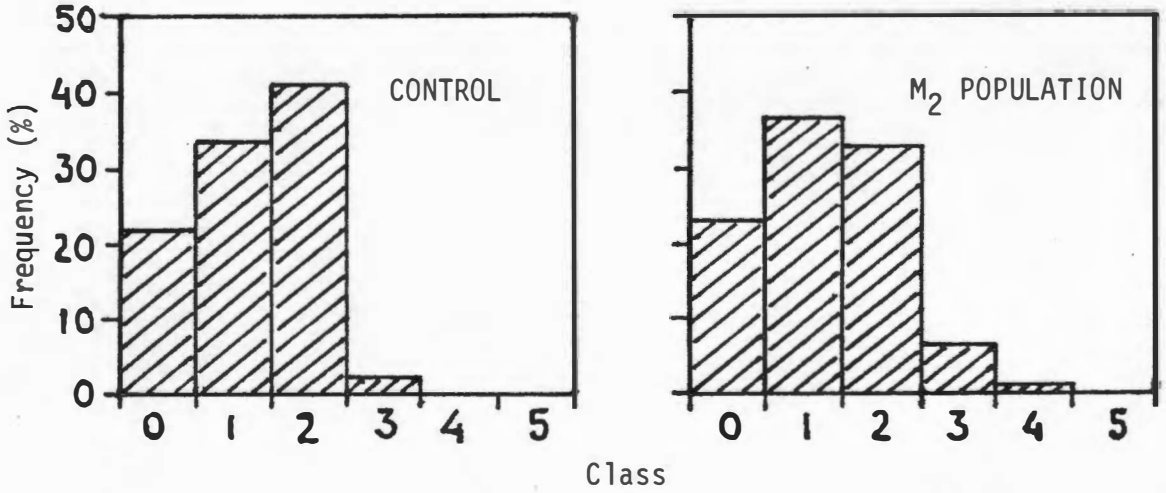


Fig. 3. Frequency distribution of the control and M_2 population of 'Forrest' variety, based on the standard classification.

TABLE 3

CONTINGENCY TABLE OF THE CONTROL AND M_2 POPULATIONS OF 'FORREST' VARIETY BASED ON THE STANDARD CLASSIFICATION

Class*	No. of Galls Per Plant	Observed Frequency: Control Population	Observed Frequency: M_2 Population	Total
0	0	9	21	30
1	1-2	14	34	48
2	3-10	17	30	47
3	11-30	1	6	7
4	31-100	0	1	1
5	>100	0	0	0
Total		41	92	133

*The last three classes were combined to meet the condition for the validity of the chi-square frequency test that the smallest expected frequency should be at least 1.

Chi-square = 1.97 ($0.50 < P < 0.60$).

riggs (1970) reported the results of their studies on the induced mutations in Lespedeza stipulacea in which the resistance of 77 irradiated strains to root-knot nematodes were tested. They found that relatively high proportions of these strains were resistant. Such information is lacking for soybeans. More studies on this subject are obviously needed to obtain information for a sound background of the use of induced mutations in plant breeding.

CHAPTER V

CONCLUSIONS

The results suggest that the variability of resistance to root-knot nematodes in 'Essex' was significantly broadened by the EMS treatment. There is a good probability of selecting individual plants with a higher level of resistance from the M_2 population of 'Essex.' In 'Forrest' the EMS treatment did not produce significant changes of the distribution of individuals with respect to the level of resistance.

By inference it is suggested that the probability of improving resistance through induced mutations in susceptible varieties is greater than the probability of breaking resistance which has been established.

Further studies on this subject by using other varieties with different levels of resistance are needed to affirm this conclusion.

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