Induction, detection and isolation of temperature-sensitive lethal and/or sterile mutants in nematodes. I. The free-living nematode *Caenorhabditis elegans* [1]

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SUMMARY

Applying a series of techniques intended to induce, detect and isolate lethal and/or sterile temperature-sensitive mutants, specific to the self-fertilizing hermaphrodite nematode *Caenorhabditis elegans*, Bergerac strain (Abdulkader & Brun, 1976), 25 such mutants have been found. Optimal conditions for the application of mutagenic treatment and the detection of such mutations are discussed.

RÉSUMÉ

Introduction, detection et isolement de mutants thermosensibles létaux et/ou stériles chez les nématodes. I. Le nématode libre Caenorhabditis elegans.

Grâce à l'élaboration d'un protocole original de détection et d'isolement des mutations thermosensibles létales et/ou stériles, spécifique du nématode hermaphrodite autofécond *Caenorhabditis elegans*, souche Bergerac (Abdulkader & Brun, 1976), 25 d'entre elles ont été obtenues. Les conditions optimales de traitement par l'agent mutagène et de détection de telles mutations sont discutées.

The prolificity of nematodes, whether parasitic or free-living, is one of their most spectacular characteristics. Applied biologists cannot fail to be intrigued by this phenomenon : those who are interested in phyto or zooparasitic species seek to limit it, while those dealing with soil fauna wish to encourage it.

As a result, the study of nematode reproduction, and particulary of gametogenesis and development, is crucial. Thus, a comparative study of these functions, on the one hand in normal individuals, on the other hand in temperature-sensitive (ts) mutants, must prove extremely interesting. This type of analysis has, in fact, been most fruitful in the understanding of multiplication, not only in microorganisms (Edgar & Lielausis, 1964 ; Fattig & Lanni, 1965 ; O'Donovan, Kearney & Ingraham, 1965 ; Condon & Ingraham, 1967 ; Skarvronskaya *et al.*, 1971), but also in multicellular plants and animals. Among the latter, attention is drawn to the results of studies among the insects, eg. *Drosophila* (Suzuki *et al.*, 1967 ; Baillie, Suzuki & Tarassoff, 1968 ; Tarassoff, 1970 ; Holden, 1973 ; Wright, 1973 ; Gans, Audit & Masson, 1975), *Habrobracon* (Smith, 1967 ; 1968 ; 1971) and *Musca domestica* (McDonald & Overland, 1972).

This success is based particularly on the fact that ts mutants, whose reproduction at permissive temperature is the same as in the wild-type, show disrupted gametogenetic or developmental

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activities when they are exposed to extreme temperatures (non-permissive temperatures) at a certain stage in their life cycle (temperature-sensitive period). The effects of the non-permissive temperature may vary, leading to sterile and/or lethal organisms.

In this study, we describe the conditions which have enabled us to obtain and isolate, in the laboratory, sterile and/or lethal ts mutants of the free-living soil nematode *Caenorhabditis elegans*, Bergerac strain. The protocols to be described here are also applicable in the case of other nematodes of the soil fauna, and with appropriate modifications, to phytoparasitic nematodes.

Materials and Methods

Characteristics and culture conditions of the strain used

The wild-type of the self-fertilizing protandrous hermaphrodite nematode *C. elegans* used in this work is native of Bergerac. Since 1944, it has been raised in the laboratory, at 18 °C, on a xenic agar medium, either in individual cultures, or in mass cultures (Brun, 1966 ; 1967). In the first case, a single nematode is placed on a drop of nutritive agar medium (Brun, 1966) set on a histological microslide (ten slides per glass-staining dish).

In the second case, the nutritive medium is poured into a 6 cm diameter (pyrex) Petri dish, to which are added at least ten nematodes. In all the quantitative and qualitative studies carried out in the course of this work, individual cultures have been used, mass cultures being used only for the maintenance of the strain and the various mutants isolated. In certain experimental modalities semi-mass cultures have been used. In this case, a drop of nutritive medium on a histological slide receives not a single individual but ten nematodes, their ages varying from egg to adult.

Previous work has shown that, by self-fertilization, hermaphrodites produce almost exclusively hermaphrodites. Exceptionally however, and with a frequency of 0.001, a male is produced (Nigon, 1949).

Thermic conditioning of nematodes and selection of non-permissive temperature

The usual temperature for raising the wild-type (18 °C) was chosen as permissive temperature. At this temperature, the wild-type nematodes reach female sexual maturity when they are four day-old and then lay eggs for a least five days (Brun & Lebre, 1968). At 24 °C, they reach sexual maturity in only 2 1/2 days, but then do not lay for more than two days.

The choice between 24 °C and 26 °C as non-permissive temperature was made by incubating wild-type eggs, laid at 18 °C by four day-old hermaphrodites and raised at this temperature, at either 24 °C, or 26 °C. In each case, their viability was determined, then the fecundity of those which developed into adults, and similarly for their descendants (when these existed).

The manipulations were as follows :

— at 18 °C, adult hermaphrodites 4 1/2 days old lay eggs on the surface of drops of nutritive agar medium (these hermaphrodites were descended, by self-fertilization, from wild-type hermaphrodites kept at 18 °C). Eggs were collected one hour after ovideposition, at 24 °C and 26 °C. In this manipulation : 1) only eggs which appeared granular and dark brown when viewed under a binocular dissecting microscope ($\times$ 160), i.e. between the 30 blastomeres and coeloblastula stages, were collected and incubated, 2) eggs were transferred on to the drops with a needle.

— after 2 1/2 to 3 days, the cultures at 24 °C and 26 °C were observed. The total number of living animals, and among them, the number of adults were determined. If these adults reproduced, their fecundity was determined as follows : they were first put into individual cultures, then transferred to new drops after one day. Then, 3 days after being put into cultures, the number of adult descendants on each of the 2 drops was determined. In this way, the fecundity at 24 °C has been examined for several successive generations.

All the temperature changes during the rearing of the nematodes were made by transferring the culture recipients from one thermostatically controlled incubator to another (Brun, 1966). All manipulations were made at room temperature (19 °C $\pm$ 1 °C) under a sterile hood.

Treatment with the chemical mutagen

To induce ts mutations, the alkylating-agent ethyl-methane-sulfonate or E.M.S. (1), dissolved in a 0.1 M potassium phosphate buffer solution (pH = 7), was used at two concentrations : 0.05 M and 0.1 M. Two day-old larvae, (which corresponds to the second larval stage) raised at 18 °C, were placed in the mutagen for different lenghts of time :

- 7, 10 and 13 hours for the 0.05 M concentration,
- 4, 7 and 10 hours for the 0.1 M concentration.

In all cases, the treatment was made at room temperature (19 °C ± 1 °C) according to the following modalities : L_2 larvae, all the same age and issued from semi-mass egg cultures, (twenty eggs per drop) were immersed in the mutagenic solution. At the end of the treatment, they were removed and immediatly transferred on to a drop of nutritive agar medium where they were cultivated in semi-mass (ten larvae per drop). As a control, similar L_2 larvae were immersed in an E.M.S. free buffer solution.

The experimental protocol for induction, detection and isolation of ts mutants is given in Figure 1.

From each E.M.S.-treated hermaphrodite, a group of at least 30 adult F_2 descendants was collected and placed on drops of medium to lay eggs for one day. This group was then discarded. Four days later, young F_2 adults were randomly collected, cultured individually for one day at 18 °C, and then transferred to other drops of medium and incubated at 24 °C. Five days after maintaining of the F_2 at 18 °C (thus, 4 days after the shift of the F_2 nematodes and their incubation at 24 °C), F_3 descendants of each F_2 animal were sought, both at 18 °C and 24 °C. Existence of fertile adults at 18 °C and their absence at 24 °C are evidence for the presence of a lethal ts mutant. A ts sterile mutant is detected by the presence of adults at both 18 °C and 24 °C, but at 24 °C adults do not lay eggs, or lay eggs which fail to hatch. The fact that other animals, brothers of these putative mutants, are kept in culture at 18 °C enables the abnormal form, detected by the above method, to be isolated.

After being reared in individual cultures at 18 °C generations (always taking animals from among the descendants of a single nematode, in order to have isogenic strains) the ts animals isolated in the F_3 produced F_4 descendants after two generations. The latter were retested as follows : two groups of ten young adults, all descendants of one F_4 animal, were reared, one group at 24 °C, the other at 18 °C. At the same time, F_2 eggs, laid at 18 °C by F_4 adults, were incubated on drops of medium (minimum 60 eggs) at 24 °C. These cultures were observed after 5 days. If the 24 °C retest was positive, i.e. either absence of fertile F_4 individuals or absence of F_4 adults, the "sister" strain maintained at 18 °C was considered to be a ts mutant, sterile or lethal respectively.

Rearing of ts mutants for experimental use

The different ts mutants were reared in individual cultures for at least 10 generations before being subjected to physiological or genetic studies. Thus, each generation consisted of the descendants of a single nematode from the preceding generation.

Results

Viability and fecundity of the wild-type strain of C. elegans

At 26 °C

As shown in Table 1, 74% of the eggs of the wild-type *C. elegans*, laid at 18 °C and incubated at 26 °C, developed, hatched and reached sexual maturity (the development time, egg → adult, being of the order of 2 1/2 days) However, although these animals appeared normal and moved normally, they were completely sterile.

(1) The choice of E.M.S. comes from its recognized effectiveness in this area on *Drosophila* (Suzuki *et al.*, 1967 ; Suzuki & Procunier, 1969 ; Suzuki, 1970).

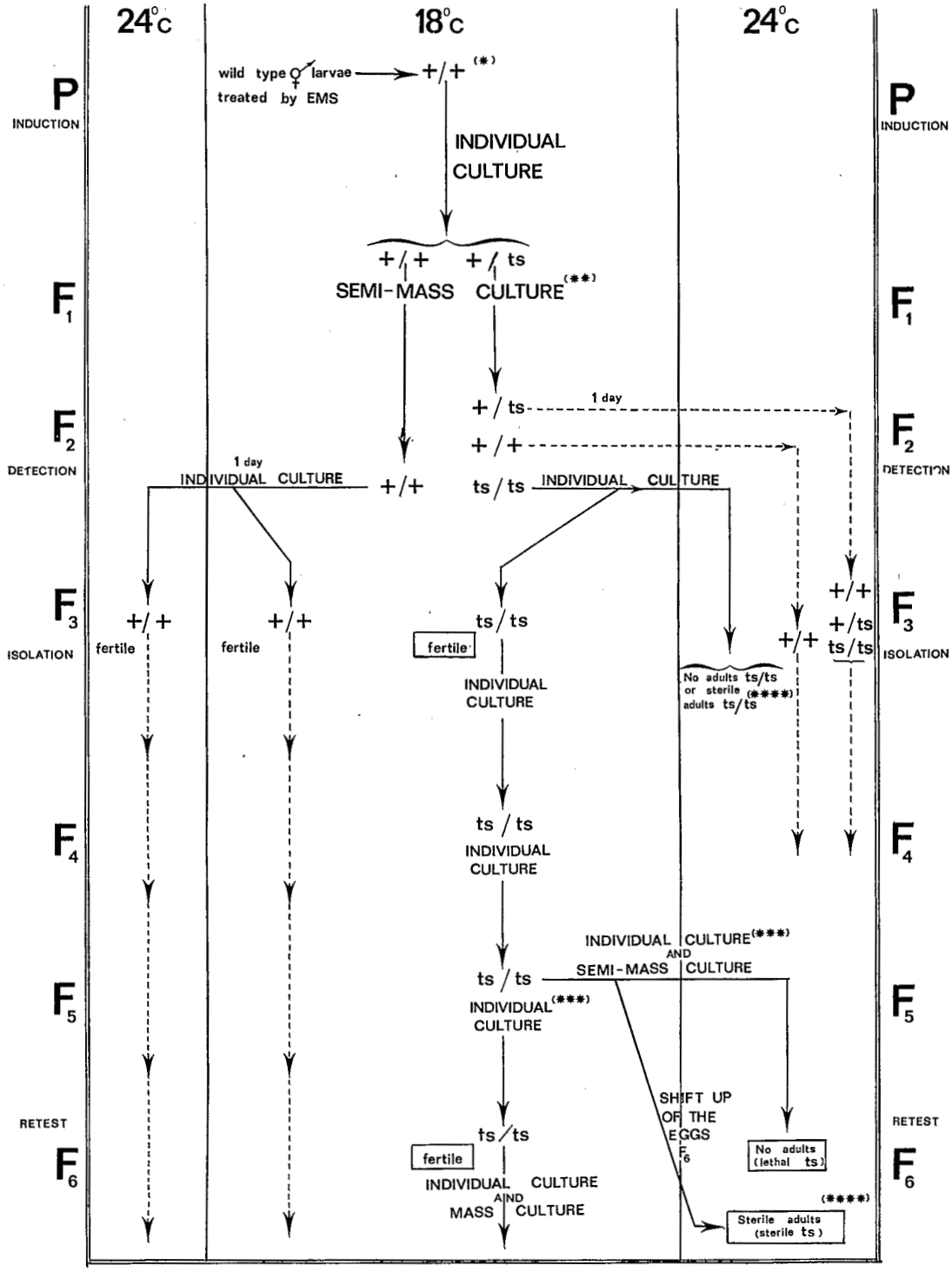

Fig. 1 : Experimental protocol for the induction, detection and isolation of temperature-sensitive sterile or lethal mutants in *C. elegans*. (*) : The genotypes mentioned in this table are all adult hermaphrodites, characterized by the presence of vulva. (**) : 30 F1 adults (minimum) are divided into lots of ten and put in virgin nutritive medium. (***) : Ten F5 per culture. (****) : adult animals which do not lay eggs or lay eggs which fail to hatch.

Table 1

Viability of the eggs of *C. Elegans* Bergerac wild-type strain at 26 °C and 24 °C.

Average viability of an egg at 26 °C $= \frac{766 + 13}{1\,050} = 0.742$. Confidence interval : 0.742 ± 0.053.

Average viability of an egg at 24 °C $= \frac{336 + 4}{450} = 0.755$. Confidence interval : 0.755 ± 0.039.

Temperature	*Number of eggs*	*Observation after a time equal to :*	*Number of survivors*		*Observations of the descendants at 26 °C of the adults*
			Larvae	*Adults*	
26 °C	150	2 d. 12 h	2	099	Very few abnormal eggs in the body.
	150	2 d. 12 h	1	123	Some abnormal eggs in the body.
	150	2 d. 15 h	4	095	Rare abnormal eggs in the body + some abnormal eggs on the drops of nutritive medium.
	150	2 d. 15 h	2	107	
	150	2 d. 15 h	3	116	idem
	150	2 d. 22 h	1	122	idem
	150	2 d. 22 h	0	104	idem
	Total 1 050		13	766	
24 °C	150	2 d. 16 h	1	118	Numerous laid eggs.
	150	2 d. 18 h	2	107	Numerous laid eggs + some larvae.
	150	2 d. 18 h	1	111	Numerous laid eggs + some larvae.
	Total 450		4	336	

Indeed, the small number of eggs laid never hatched. Besides, examination with dissecting microscope (× 160) revealed that they were all abnormal. One category appeared to be a cytoplasmic mass, lacking in consistency and enclosed in a membrane, while others were spherical. At the centre of all these eggs was a dark brown zone surrounded by a paler ring.

At 24 °C

As far as the viability is concerned (Tab. 1), the result was similar to that obtained at 26 °C, of the order of 75%. A close analogy also holds between the development times, egg → adult, which in both cases was 2 1/2 days (Tab. 1).

On the other hand, at 24 °C, the animals were still fertile. Fecundity of F_0 hermaphrodites was of the order of 55, and those of the descendance F_1, F_3 and F_4, cultivated at 24 °C, were 34.5, 48.9 and 49.3 respectively (Tab. 2). In addition, these animals could be maintained in culture at 24 °C.

Table 2

Fecundity of *C. Elegans* Bergerac of wild-type strain at 24 °C. F_0 = 1st generation at 24 °C (eggs laid at 18 °C, then shifted to 24 °C → adults F_0) ; F_1 = 2nd generation at 24 °C ; F_3 = 4th generation at 24 °C ; F_4 = 5th generation at 24 °C

Generation	*Sample size*	*Average fecundity*
F_0	81	55.00 ± 2.75
F_1	70	34.51 ± 3.59
F_3	44	48.91 ± 6.21
F_4	31	49.37 ± 9.94

Detection and isolation of sterile and lethal ts mutants

Sixty putative mutants have been detected following a systematic examination of the F_3 descendants of 1404 F_2 adults, taken in varying numbers depending on the different treatments. A retest of these 60 strains after two generations has enabled the definitive isolation of 25 ts mutants : 13 steriles and 12 lethals.

Frequency of ts mutants in the F_2 : estimation of the ts mutation rate

As is shown in Table 3, the mutation frequency in the F_2 varied from 0.7 to 2.13%, according to the different mutagenic treatments. It was not possible, however, to establish whether the differences in these frequencies were statistically significant, since any decision would be of doubtful value because of the low sample size (378) for the test at the 0.05 M E.M.S. concentration.

As far as estimation of the gametic mutation rate P is concerned, where :

$$P = \frac{\text{Total number of ts mutated gametes,}}{\text{total number of gametes produced}}$$

the following simple relation is a good approximation :

$$P = 2\,f$$

where f is the frequency of ts/ts individuals in the F_2, i.e. :

$$f = \frac{\text{number of mutants obtained.}}{\text{number of worms tested}}$$

Indeed, if one admits that :

a. treatment of L_2 larvae induces mutations essentially in the gonia and that it is equally likely that these give rise to spermatozoa or oocytes ;

b. mutations affect the chromosomes ;

c. probability of induction of 2 ts mutations in the same gamete is extremely small, thus negligible ; *a fortiori*, in the same chromosome ;

d. F_1 individuals are all equally fertile ;

e. eggs of ts/ts individuals have the same viability at 18 °C as other genotypes, and

f. fusion of 2 F_2 ts-mutated gametes is very rare, thus negligible,

then the frequency of ts/ + heterozygous animals in the F_1 is 4 times that of ts/ts homozygous animals in the F_2 and therefore equal to 4 f. But, on the other hand, the frequency of ts/ + heterozygous nematodes is only twice that of ts-mutated F_1 gametes, i.e. :

$$P = 1/2\,(\text{frequency of ts/+ } F_1 \text{ worms}) = \frac{4f}{2} = 2f.$$

Table 3
ts mutation frequency in the F_2 and estimation of mutation rate.

	Molarity and duration of E.M.S. treatment					
	0.05 M × 7 h	*0.05 M × 10 h*	*0.05 M × 13 h*	*0.1 M × 4 h*	*0.1 M × 7 h*	*0.1 M × 10 h*
Number of F_2 tested	144	162	72	422	424	180
Number of mutants isolated	1	2	1	9	9	3
Mutant frequency in F_2	06.9%	1.23%	1.38%	2.13%	2.12%	1.63%
Mutation rate	1.38%	2.46%	2.77%	4.26%	4.24%	3.27%

RESEARCH INTO OPTIMAL CONDITIONS FOR THE APPLICATION OF MUTAGENIC TREATMENT : EFFECTS OF EMS ON THE ANIMALS TREATED

The number of adults obtained 3 days after larvae had been subjected to different treatments was determined. Viability of the larvae treated was defined as the number of adults obtained divided by the number of larvae treated.

Once they reached the adult stage, the treated worms were cultured individually for 1 1/2 days, then transferred to new drops. Five to 5 1/2 days after being put into cultures, the number of adult descendants of the treated animals (i.e. their fecundity) was determined.

As shown in Table 4, the toxic effect of the alkylating agent is immediatly obvious. Statistical analysis, by means of the ε test (Table 4A), shows that this lethal effect increases with E.M.S. concentration, and for the 0.1 M concentration, with time.

The fecundity of treated worms (Table 5) was always significantly lower than that of control worms (Table 5A). In addition, worms treated with a 0.1 M E.M.S. solution showed a significant decrease in fecundity, dependant on the treatment time.

This decrease in fecundity may be explained as follows : a low concentration (0.02 M) or a 0.1 M E.M.S. treatment for only 4 hours, resulted in a certain inhibition in gonia multiplication processes and in gametic differentiation. On the other hand, prolonged action at the high concentration 0.1 M for 7-10 hours caused, in addition, gonial and gametic lethality which increases with treatment time.

Discussion

SELECTION OF NON-PERMISSIVE TEMPERATURE

Theoritically, a large difference between the permissive and non-permissive temperatures increases the probability of detection of ts mutants (Suzuki, 1970). However, in practice, this interval has to be limited. Indeed a very high non-permissive temperature greatly reduces the viability and fecundity of the wild-type strain, at the extreme reducing these parameters to zero, and thus the wild-type can no longer be distinguished from the mutants.

In wild-type *C. elegans*, Bergerac strain, an increase in temperature from 24 °C to 26 °C, did not decrease its viability but prevented its reproduction. Thus, 26 °C cannot be used for mutant detection, and 24 °C was adopted as non-permissive temperature. In addition, it does not seem necessary to examine the effects of 25 °C, used as non-permissive temperature in the case of the Bristol strain of *C. elegans* (Hirsh & Vanderslice, 1976), since at 24 °C, the viability of the wild-type Bergerac is already reduced by 10% with respect to 18 °C, and its fecundity by more than 50%. Indeed, Fatt and Dougherty (1963) have shown that the Bristol strain is more heat-resistant than the Bergerac.

Table 4
Viability of larvae treated by E.M.S.

	Type of E.M.S. treatment						
	E.M.S. concentration and treatment time						
	0.05M × 7 h	*0.05M × 10 h*	*0.05M × 13 h*	*0.1M × 4 h*	*0.1M × 7 h*	*0.1M × 10 h*	*Control : 13 h in E.M.S.-free buffer solution*
Number of treated larvae	240	240	168	200	240	240	50
Number of adults obtained	166	162	121	129	123	91	45
Viability	0.691	0.675	0.720	0.645	0.512	0.379	0.900

Table 4 A

Comparison of the viability of larvae treated by E.M.S.
ε-test (comparison of percentages) Critical value for 5% significance level = 1.96
* Significant difference

Types of treatment and viability	*Type of treatment and viability*						
		E.M.S. concentration and treatment time					
	Control : 13 h in E.M.S.-free solution	*0.05M × 7 h*	*0.05M × 10 h*	*0.05M × 13 h*	*0.1M × 4 h*	*0.1M × 7 h*	*0.1M × 10 h*
	0.900	0.691	0.675	0,720	0.645	0.512	0.379
Control : 13 h in E.M.S.-free solution 0.900		3.010*	3.202*	2.618*	3.506*	5.049*	6.713*
0.05 M × 7 h 0.691			0.392	0.621		4.009*	
0.05 M × 10 h 0.675				0.975			6.490*
0.1 M × 4 h 0.645						2.797*	4.121*
0.1 M × 7 h 0.512							2.939*

Table 5

Fecundity of animals treated by E.M.S.

	Type of E.M.S. treatment						
	E.M.S. concentration and treatment time						
	0.05 M × 7 h	*0.05M × 10 h*	*0.05M × 13 h*	*01.M × 4 h*	*0.1M × 7 h*	*0.1M × 10 h*	*Control : 13 h in E.M.S.-free buffer solution*
Fecundity of treated animals	80.26 ± 9.90	81 ± 8.02	76.30 ± 10.00	38.15 ± 12.33	52.76 ± 10.62	36.59 ± 9.75	139 ± 11.09
Sample size	30	33	32	26	22	22	30

Table 5 A

Comparison of the fecundity of animals treated by E.M.S.
t-test (comparison of means). Critical value for 5% significance level = 1.96
* Significant difference.

	Type of treatment and fecundity						
		E.M.S. concentration and treatment time					
Type of treatment and fecundity	*Control: 13 h in E.M.S.-free buffer solution*	*0.05M × 7 h*	*0.05M × 10 h*	*0.05M × 13 h*	*0.1M × 4 h*	*0.1M × 7 h*	*0.1M × 10 h*
	139 ± 11.09	80.26 ± 9.90	81 ± 8.02	76.50 ± 10	88.15 ± 12.33	52.76 ± 10.82	36.59 ± 9.75
Control 139 ± 11.09		11.176*	12.076*	11.858*	8.705*	15.713*	19.406*
0.05 M × 7 h 60.26 ± 9.90			0.168	0.755		5.340*	
0.05 M × 10 h 81 ± 8.02				0.994			10.146*
0.1 M × 4 h 88.15 ± 12.32						6.069*	9.190*
0.1 M × 7 h 52.76 ± 10.82							3.140*

Nevertheless, even though 26 °C cannot be used as non-permissive temperature in the detection of ts mutants, due to the complete sterility of the wild-type strain, the viability of this strain is still very high (74%) at 26 °C enabling genetic and physiological studies of the lethal mutants obtained to be carried out.

Induction and detection of ts mutations in nematodes

At 0.05 M and 0.1 M concentrations, and for treatment times from 4 to 13 hours, E.M.S. treatment of *C. elegans* larvae induces lethal and/or sterile ts mutations at 24 °C, by its action on the L_2 larvae. The mutation rate is always highest for the 0.1 M concentration (3 to 4%). Although at this concentration there are not significant differences in the mutation rate, whether the E.M.S. treatment is used for 4, 7 or 10 hours, short treatments (4 hours and 7 hours) are to be recommended for nematodes.

Indeed, the greatly reduced fecundity of the animals subjected to long treatments on the one hand (Table 4), and of their F_1 descendants on the other, prompts us to think that, in such conditions, numerous non-conditional lethal and/or sterile mutations are induced in the genome. Inevitably, this must lead to the death of a certain number of ts mutants.

In contrast with experimental protocols used

to obtain ts mutants in *Drosophila* (Suzuki, 1967 ; Fattig, 1972 ; Ayles, 1973 ; Gand, 1975), our methods (Abdulkader & Brun, 1976), summarized in Figure 1, allow detection in a single step of all sterile and/or lethal ts mutations occurring in any part of the genome. However, this technique requires a complementary genetic study to define, for each mutation, the chromosomal localization and precise genetic determinism.

Influence of EMS concentration and of the difference between permissive and non-permissive temperatures on the mutant frequency and detection possibilities

In the Bristol strain of *C. elegans*, Hirsh and Vanderslice (1976), following a detection protocol very similar to ours, obtained results not significantly different, as far as mutation rate and mutant frequency in F_2 are concerned. However, they used a 0.05 M E.M.S. instead of 0.1 M, and as permissive and non-permissive temperatures, 16 °C and 25 °C instead of 18 °C and 24 °C, i.e. a 9 °C difference instead of 6 °C.

Can one conclude, then, that in *C. elegans* the frequency with which ts mutants can be detected is a function of the difference between the permissive and non-permissive temperatures ?

In terms of our present knowledge in this field, any answer to this question would be premature, owing to the distinct differences between the Bergerac and Bristol strains, particularly in their fecundity and heat-tolerance. Decisive experimental arguments could only be given after precise comparative studies have been carried out on the two strains. Such studies are not the easiest to make, since the detection of ts mutants is subjected to constraints which are difficult to bypass : one of the most compelling is the necessity to know, simultaneously, the behaviour of the F_3 at permissive and non-permissive temperatures. This, for example, prevents the use of 13 °C as permissive temperature, even though the animals reproduce normally in such conditions. Indeed, at 13 °C, *C. elegans* takes 8 days to reach the adult stage, while at 24 °C, 2 1/2 days is sufficient. Thus, even though is would be possible to characterize F_3 individuals obtained at the permissive temperature, "sister" cultures transferred to 24 °C would consist not only of senile F_3 but also of F_4 and F_5.

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