A Study on Spontaneous Chromosome Fragmentation in *Vicia sativa* Linn.

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Introduction

The genus *Vicia* has become a classical material for cytologists due to continued researches on the members of this genus by various workers since long back (Heitz, 1931; Bhaduri, 1940; Levan and Lotfy, 1950 etc.). In addition to working out the cytology, the members have also been tested to detect their susceptibility towards chemicals, and considerable facts of fundamental importance have emerged out from these researches. Easy germination, low number and presence of easily detectable chromosomes with simple morphology are the factors responsible for this genus being an ideal material for cytologists.

Recently while studying the effect of chemicals on *Vicia faba*, Levan and Lotfy (1950) have come across certain interesting cases of spontaneous fragments in certain preparations of their plants.

The fragments have always been found to arise out of symmetrical chromosome changes affecting both the chromatids and mostly the heterochromatic regions. They assume that the breakage starts while the cells are still dormant that is, at a very early stage during soaking. As seedlings germinated in saw-dust with pre-soaking in water contain fragments much more in number than those germinated without pre-soaking, they claim that the soaking in water during the early stages favours fragmentation.

It has been suggested that "the partially anaerobic conditions during the twenty-four hours in which the dormant vital processes are awakened may involve a change of the metabolism with formation in high concentration of some metabolites, finally leading to chromosome breakage".

In connection with the study of the possibilities of the use of chemicals in chromosome analysis in dicotyledonous plants, *Vicia sativa* is being used here as an experimental material. As in the case of Levan and Lotfy (1950) in some of the control preparations, spontaneous fragments are noticed. How far such spontaneous fragments, in this species of *Vicia*, are due to water-relation of the plants during the early stages has been tested during the present investigation. Further, a detection of the specialised region of the chromosome, if any, susceptible to such breakage has also been included within the scope of the study. An attempt has been given to collect data on the frequency of fragments in the different stages and the fate of such bodies in

cell-division. The role played by chemicals causing chromosome abnormalities in accelerating or retarding this fragmentation frequency has further been brought out in the present work.

It was considered that if the cause of such chromosome-disbalance occurring spontaneously be found out, some important problems of chromosome-metabolism would be solved and unknown properties of their structure revealed.

Method and observations

The materials for the present investigation were obtained from the Leguminous plant, *Vicia sativa* Linn.

The plant is cultivated intermixed with pulses. It is a herbaceous annual of the tropical climate, and as such, could be grown suitably in the experimental plot attached to the laboratory. Seeds were obtained from the field along with pulses. Seeds, if artificially stimulated, are capable of germination at any time of the year.

The aim of the present investigation being a study of the spontaneous chromosome fragmentation, as stated earlier, several controls had to be prepared. Two lots of air-dry seeds—

i. With twenty-four hours soaking in tap water just before germination, and

ii. Without soaking in water before germination

were allowed to germinate in moist saw-dust in the laboratory as well as in the field. Healthy root-tips, when about 0.3 cm. long, were collected every day from both the lots beginning from the 1st day of germination and continuing upto the tenth day.

The peak period of division of the species was found to be between 10 A.M. and 1 P.M.

Fixatives. Fixatives used could be classed under two different categories, namely, those having no fragmentation-inducing so-called mutagenic property and as such used for the purpose of detection of spontaneous chromosome fragments; and secondly those which are mutagenic in nature, that is endowed with the property of inducing fragmentation, and hence tested for their accelerating effect on fragmentation. The term mutagenic has been used in a broad sense, including even the properties of causing chromosome breakage. Fixatives used were the following:

A. Fixation in the fluid containing mutagenic chemicals

i. Acetic-alcohol fixative—

Glacial acetic acid....1 part

Absolute alcohol1 part

ii. Lewitsky's fixative—

1% Chromic acid1 part

10% Formalin1 part

B. Fixation in fluid containing mutagenic chemicals

- i. Temporary phenol treatment—
(Sharma and Bhattacharjee, 1954)
- ii. Pre-treatment of phenols followed by fixation in
1% Chromic acid1 part
10% Formalin1 part

Procedure of different methods

Acetic-alcohol treatment. Fresh healthy root-tips and some cross sections from both the lots (as described before) were fixed in acetic-alcohol (1:1) for forty-five minutes at room temperature (26°C). The tips were next hydrolyzed for fifteen minutes at 58°C followed by Feulgen squashing in 45% acetic acid as usual. Thereafter sealing with paraffin was done as usual.

Temporary phenol treatment. Root-tips were fixed in phenols of suitable concentrations for three hours in cold (5–12°C) as well as in room temperature (26°C).

Phenols used :

- i. Phloroglucinol....0.05 M to 0.1 M
- ii. Resorcinol0.005 M to 0.1 M

Subsequent to fixation, the tips were hydrolyzed in N·HCl for fifteen minutes at 58°C followed by Feulgen squashing as usual. The slides were then sealed with paraffin.

Pre-treatment in phenols followed by fixation in chromic formalin. Root-tips were fixed in phenols for three hours, after which they were transferred to Lowitsky's fixative (1% chromic acid : 10% formalin : 1:1). After overnight fixation paraffin blocks were prepared following the usual procedure of dehydration, infiltration and embedding in paraffin.

In case of microtome ribbons, they were cut at a thickness of 14 μ and stained following the usual schedule of Newton's Crystal violet-Iodine technique. An overnight pre-mordanting in 1% chromic acid was found to be essential.

The figures were drawn at a table magnification of approximately $\times 1700$ times using a Leitz compensating eyepiece of $\times 12$ and a 1.3 apochromatic objective with a condenser of 1.4 N.A.

Microphotographs and most of the drawings were taken from temporary preparations.

In figures (1, 4 and 5) the chromosomes were drawn after spacing them apart.

Way of treatment. On the very first day of the commencement of germination, when the roots attained a length of about 0.3 cm. they were found to be suitable for observation. Roots from different lots, as mentioned in the text, that is, seeds of one with twenty-four hours pre-germination soaking in tap water and those of the other without any such pre-germination soaking in tap water were observed and the observations were continued, beginning from the very first day upto the tenth day.

Most of the preparations were made following Feulgen-squash technique, which has been found to yield be better preparations than orcein, specially in dicotyledonous plants. The procedure, as described in detail in the text, involved essentially a hydrolysis in N-HCl for fifteen minutes at 58°C. and then staining and with Feulgen solution and squashing as usual.

Figs. 1-14. Fragmentation in *Vicia faba* root tips. 1-10, without pre-germination soaking (acetic alcohol treatment), showing different mitotic stages with different numbers of fragments. 11 & 12, with 24 hours pre-germination soaking (acetic alcohol treatment), showing prophase with 2 & 3 fragments respectively. 13-14, without pre-germination soaking (resorcinol treatment), showing metaphase with 4 & 2 fragments respectively.

Method of taking counts. In determining the frequency of fragmentation only those cells with clear divisional stages, viz., prophase, metaphase, anaphase and even early telophase were taken into account. Caution was particularly taken to avoid duplication in count of a particular cell. The possibility of such errors was eliminated by recording the reading of the vernier scale of the microscope stage and simultaneously taking corresponding counts of cells at that particular reading. In this way all the cells with clear divisional stages were counted and the corresponding number of fragments noted. The

whole process of counting was done under the oil-immersion lens in order to minimise the error as regards detection of the number of fragments.

Number of fragments in each cell and divisional stages. Number of fragments observed in different cells was not constant, but rather a variable number of fragments was noted. The number of fragments were one, two, three and even four in some cases. A peculiar behaviour, noted in all the sets of the experiment, was that the maximum number of fragments within a cell did not exceed four. Cells with two fragments were found to occur in maximum frequency.

An important result of observation which needs mention is that fragments were found in all the different stages of the divisional cycle, viz., prophase, metaphase, early anaphase, anaphase and even early telophase. The frequency was found in the prophase stage to be maximum (Figs. 1-14).

Micronucleus formation

Micronucleus was found to occur in some of the resting cells, frequency being very low. In all cases micronucleus was found to be always provided with a nucleolus. Number of micronuclei, when present in a cell, was always found to be one.

In order to study the frequency of fragmentation in different zones, viz., cortical and central regions, cross-sections of root-tips were smeared after Feulgen staining. Maximum frequency was noted in the cortical region while in the central region it was very low, as is evident from the Table (No. 10).

The experiments were conducted upto the tenth days after germination. During the early period, the frequency of fragments was found to be rather high, which gradually decreased and ultimately the fragments completely disappeared. On the ninth days and afterwards, fragments and micronuclei were not observed.

Results so far obtained are tabulated below :

Table 1. Shows gradual decrease of fragments and micronuclei with age

Days of observation	Fragmentation frequency	Micronucleus frequency
1st day	High	High
2nd "	"	"
3rd "	"	"
4th "	Low	Low
5th "	"	"
6th "	"	"
7th "	Very low	"
8th "	Rare	Absent
9th "	Absent	"
10th "	"	"

Table 2. Fragmentation without pre-germination soaking (Acetic-alcohol treatment)

No. of observation	No. of cells noted in each field of observation	No. of cells with fragments	Stages	No. of fragments in each cell
1	23	1	Metaphase	2
2	50	2	i. "	2
			ii. "	1
3	18	1	Prophase	1
4	38	Nil	—	—
5	14	2	i. Anaphase	2
			ii. Prophase	2
6	16	1	"	2
7	5	1	"	1
8	32	Nil	—	—
9	15	2	i. Anaphase	2
			ii. Prophase	1
10	13	1	Metaphase	1
11	27	Nil	—	—
12	19	1	Telophase	2
Total	270	12		

* Number of cells=Number of cells with clear divisional stages.

Out of two hundred and seventy cells with clear divisional stages twelve cells are found to contain fragments. Maximum number of fragments is found to be three. Maximum frequency of fragments has been noted in prophase.

Table 3. Frequency of fragmentation without pregermination soaking (Acetic-alcohol treatment)

*Total No. of cells observed	Total No. of cells with fragments	Percentage
270	12	4.44%
270	Total No. of prophases with fragments	
	5	1.83%
270	Total No. of metaphases with fragments	
	4	1.48%
270	Total No. of anaphases with fragments	
	2	0.74%
270	Total No. of telophases with fragments	
	1	0.37%

* Number of cells=Number of cells with clear divisional stages.

Figs. 1-5. Fragmentation in root-tips of *Vicia sativa* Linn. 1-3, prophase, metaphase and telophase showing the presence of fragments indicated by arrow. 4-5, micronucleus associated with a normal nucleus (arrow indicates micronucleus).

Table 4. Fragmentation with 24 hours pre-germination soaking
(Acetic-alcohol treatment)

No. of observations	*No. of cells noted in each field of observation	No. of cells with fragments	Stages	No. of fragments in each cell
1	17	1	Prophase	2
2	36	2	i. "	1
			ii. Metaphase	3
3	43	3	i. Prophase	2
			ii. Anaphase	1
			iii. Prophase	2
4	30	Nil	—	—
5	9	1	Metaphase	2
6	15	1	"	3
7	20	Nil	—	—
8	12	1	Prophase	1
9	23	1	Telophase	2
10	29	Nil	—	—
Total	234	10		

* No. of cells=Number of cells with clear divisional stages.

Out of two hundred and thirty-four cells only ten contained fragments, maximum number being three. Frequency of fragments was high in prophase.

Table 5. Frequency of fragmentation with 24 hours pre-germination soaking (Acetic-alcohol treatment)

*Total No. of cells observed	Total No. of cells with fragments	
234	10	4.27%
	Total No. of prophases with fragments	
234	5	2.135%
	Total No. of metaphases with fragments	
234	3	1.281%
	Total No. of anaphases with fragments	
234	1	0.427%
	Total No. of telophases with fragments	
234	1	0.427%

* No. of cells=Number of cells with clear divisional stages.

Table 6. Fragmentation without pre germination soaking (3 hours Resorcinol treatment)

No. of observations	"No. of cells noted in each field of observation"	No. of cells with fragments	Stages	No. of fragments in each cell
1	22	3	i. Prophase	1
			ii. Metaphase	4
			iii. Prophase	3
2	37	2	i. Metaphase	3
			ii. Prophase	2
3	27	2	i. "	3
			ii. Anaphase	2
4	54	3	i. Metaphase	4
			ii. Prophase	3
			iii. Telophase	1
5	33	2	i. Anaphase	1
			ii. "	2
6	39	2	i. Telophase	2
			ii. Prophase	3
Total	212	14		

* No. of cells observed=Number of cells with clear divisional stages.

Out of two hundred and twelve cells observed, fourteen cells showed fragments, the maximum number being four, occurring in high frequency in prophase.

Table 7. Frequency of fragmentation without pre-germination soaking
(Three hours Resorcinol treatment)

*Total No. of cells observed	Total No. of cells with fragments	Percentage
212	14	6.6%
212	Total No. of prophases with fragments 6	2.83%
212	Total No. of metaphases with fragments 3	1.415%
212	Total No. of anaphases with fragments 3	1.415%
212	Total No. of telophases with fragments 2	0.943%

* No. of cells=Number of cells with clear divisional stages.

Table 8. Fragmentation with 24 hours pregermination soaking
(Three hours Resorcinol treatment)

No. of observations	"No. of cells noted in each field of observation"	No. of cells with fragments	Stages	No. of fragments in each cell
1	19	2	i. Metaphase	3
			ii. "	2
2	17	1	"	4
3	28	3	i. Telophase	2
			ii. Anaphase	1
			iii. "	2
4	11	1	Prophase	3
5	22	Nil	—	—
6	15	2	i. Prophase	2
			ii. Metaphase	2
7	31	2	i. Prophase	1
			ii. "	3
8	24	2	i. Telophase	1
			ii. Anaphase	1
Total	167	13		

* No. of cells observed=No. of cells with clear divisional stages.

Out of one hundred and sixty-seven cells observed, thirteen show fragments, maximum being four. Frequency of fragments is high in prophase and metaphase.

Table 9. Frequency of fragmentation with 24 hours pre-germination soaking
(Three hours Resorcinol treatment)

*Total number of cells observed	Total number of cells with fragments	Percentage
167	13	7.78%
Total number of prophases with fragments		
167	4	2.39%
Total number of metaphases with fragments		
167	4	2.39%
Total number of anaphases with fragments		
167	3	1.805%
Total number of telophases with fragments		
167	2	1.195%

* No. of cells=No. of cells with clear divisional stages.

Table 10. Fragmentation in cortical and central region without soaking
(Acetic-alcohol treatment)

No. of observations	*No. of cells noted in each field of observation	No. of cells with fragments	Stages	No. of frag-ments in each cell	
C O R T I C A L R E G I O N	1	30	2	i. Metaphase	3
				ii. Prophase	2
	2	23	2	i. "	3
				ii. Anaphase	1
				"	
	3	49	3	i. "	2
				ii. Prophase	3
				iii. Metaphase	2
	4	33	1	Prophase	1
	Total		8		
C E N T R A L R E	1	21	1	Prophase	2
	2	19	Nil	—	—
	3	17	Nil	—	—
	4	11	1	Metaphase	3
	5	7	Nil	—	—
	Total		2		
G I O N					

* No. of cells=No. of cells with clear divisional stages.

Frequency of dividing cells in the central region, as may be noted, is very low.

Table 11. Frequency of fragmentation in cortical and central region without soaking (Acetic-alcohol treatment)

C O R T I C A L R E G I O N	*Total number of cells observed 135	Total number of cells with fragments 8	Percentage 5.92%
	"	Total number of prophases with fragments 4	2.96%
	"	Total number of metaphases with fragments 2	1.48%
	"	Total number of anaphases with fragments 2	1.48%
C E N T R A L R E G I O N	*Total number of cells observed 75	Total number of cells with fragments 2	2.66%
	"	Total number of prophases with fragments 1	1.33%
	"	Total number of metaphases with fragments 1	1.33%

* No. of cells=Number of cells with clear divisional stages.

Table 12. Frequency of micronuclei

No. of observations	*No. of cells noted in each field of observation	No. of cells with micronucleus	No. of micronucleus	Cells with or without nucleolus
1	70	Nil	—	—
2	63	1	1	With nucleolus
3	87	Nil	—	—
4	75	Nil	—	—
5	93	1	1	With nucleolus
6	61	Nil	—	—
7	59	Nil	—	—
Total	508	2		

Table 13. Percentage of micronuclei

Total number of cells observed	Total number of micronuclei	Percentage of occurrence
508	2	0.393%

* No. of cells observed=Number of resting cells.

Discussion

The observations put forward above clearly reveal that the fragmentation here is not conditioned by any chemical treatment. They have been noted to occur spontaneously during the early period of germination, the frequency gradually decreasing with increase in age. An acceleration has, however, been noted following treatment with phenols.

The particular stage where fragments have been noted involves different phases of nuclear division, namely, prophase, metaphase, anaphase and telophase. It is significant that the frequency of occurrence of fragments has been found to be maximum during the prophase. In experiments without pre-germination soaking, out of two hundred and seventy cells with clear divisional stages observed after first day of germination, fragments are recorded in twelve cells, frequency being 4.44%. Of these, prophases with fragments are five in number and as such frequency is as high as 1.85%. Similarly in pre-soaking cases, out of two hundred and thirty-four cells observed, ten cells contain fragments, frequency being 4.27% and of these, prophases with fragments are five in number, the frequency being as high as 2.135%. Moreover, all the fragments arise out of symmetrical breaks in view of the fact that each of them is composed of two identical chromatids. As the frequency has been noted to be maximum during prophase and also taking into consideration this double chromatid appearance of the fragments, it seems quite possible that the breakage occurs during the so-called resting stage or in the very early stage of prophase, being in all probability in the latter. It does not of course necessarily imply that the metaphase stages are not at all susceptible to breakage. The capability or breakage in this stage might differ in the degree of susceptibility to this process. The slight decrease in number noted during metaphase and later stages might be due to the fact that some of the fragments undergo degeneration in the early stage just after its formation or during the metabolic stage. That the fragments undergo degeneration is noted in experiments continuing upto the tenth day. The frequency of fragments there gradually decreases, being absent after a certain specific period.

It has been further pointed out that chemicals with mutagenic property have been applied to the root-tips for testing their accelerating property. With a certain specific dose, nearing the lower threshold concentration of activity, the frequency of fragments per cell has not been found to exceed four in number. It is of note that in case of control experiments, or more precisely, in non-treated root-tips, the number of spontaneous fragments has been found to be not exceeding three.

This number of fragments might indicate the susceptibility of certain of the chromosome parts to breakage and as such reflect a linear chemical differentiation of chromosome segments. Though innumerable data have been gathered on the mutagenic property of chemicals (Levan and Tjio, 1948;

Levan and Lotfy, 1949; D'Amato, 1950; Levan and Tjio, 1951; Levan and v. Wangenheim, 1952, etc.) none of them excepting a few (Ford, 1948; Deufel, 1952) reveal with certainty the susceptibility of certain specialised segments of chromosome.

Cases of localized chromosome breakage have recently been reported by Kihlman and Levan (1951) in *Vicia faba*. Some of the chemicals, they claim, affect first the attachment thread of the satellite in the *m* chromosome of *Vicia faba*. It is significant that in case of *Vicia sativa* there are altogether four chromosomes with secondary constrictions or satellites. Though, however, in the present case in all such cases of fragmentation, the morphology of the other chromosomes could not be worked out in detail, the number four itself needs careful attention. Though no statement with absolute certainty can be made as to the susceptibility of specific regions of chromosome to breakage, but in view of the data gathered by Kihlman and Levan (1951) as well as taking into consideration the number of fragments noted during the present investigation, one is tempted to suggest that here too secondary constrictions are mainly involved in the process. Further researches are necessary to substantiate the statement which no doubt would give a definite indication of the chemically differentiated nature of nucleolar segments. The behaviour of these regions have been shown in Papers published of the authors (Sharma and Bhattacharyyee, l.c.) to differ markedly from the other chromosome segments brought out in treatments involving certain chemicals.

In view of these points, their cycle throughout nuclear division, as well as their correlation with nucleolar synthesis, the nucleolar segments have been suggested to have a chemical make-up different from the rest of the chromosome parts (Sharma, 1955).

It has been shown that in treatment involving phenols, where the fragments have not been found to exceed four in number, the concentration applied is near the lower threshold range of activity. It must be emphasized that fragmentation involving other segments of chromosome can be caused in treatments with stronger concentration of phenols, as noted in the case of other plants. It is quite likely that in higher concentration the toxicity reaches a level in which all the chromosome segments react in a generalized way. The subtle difference in chromosome segments as regards the reactivity can thus be demonstrated only in treatments with minute dosage of chemicals thereby causing very slight toxicity.

The present observation in *Vicia sativa* differs in one respect from that of *V. faba* reported by Levan and Lotfy (1950) in that in the present case the water relation during the early period of germination has been found to exert no effect in the process of fragmentation. This has been inferred from experiments conducted here both under laboratory condition as well as in the field. It seems quite likely that the partial anaerobic conditions prevalent during the early stage of germination of the seed might result in the accumu-

lation of certain toxic chemicals responsible for causing chromosome breakage. A detailed investigation involving detection of all the constituents during the early period of germination in these cases, their intermediate products, and their effects on cell division may provide clues to the solution of the problem.

That the fragments are endowed with the capability of carrying out certain processes is evidenced by the occurrence of micronuclei in certain cases. That satellited chromosomes or chromosomes with secondary constrictions are possibly involved in the process of fragmentation have further been evidenced by the universal occurrence of nucleoli within the micronuclei, the nucleoli formation being considered by some authors as a property attributable only to chromosomes with secondary constrictions or satellites (Gates, 1942). Micronuclei, however, have not been found to be of frequent occurrence indicating that some of the fragments may degenerate without forming any micronucleus.

It is to be remembered that no specific zones of the root-tip could be observed showing fragments in their nuclei. They occur quite at random showing thereby that some sort of generalized effect, to some extent toxic, is responsible for breakage.

That this process of spontaneous fragmentation has got no role in evolution is apparent from their gradual degeneration with the increase in age of the plant. But as has been pointed out, an understanding of the cause of this phenomenon may reveal certain facts of fundamental importance regarding chromosome structure and cell metabolism.

Summary

1. Roots from two lots of air-dry seeds of *Vicia sativa* Linn., that is,
 - i. With twenty-four hours pre-germination soaking in tap water, and
 - ii. Without any such pre-germination soakingwere tested cytologically for the study of spontaneous fragmentation in the somatic tissue.
2. Chemicals having no property of causing fragmentation (so-called mutagenic) were used in techniques applied for the detection of fragments, whereas those chemicals having the property of causing fragmentation were used in some sets to study their accelerating or retarding effects on the fragmentation process.
3. Number of fragments noted were found to be one, two, three and even four, but in no case exceeding that number. Fragments were located during all the nuclear phases of the divisional cycle, namely, prophase, metaphase, anaphase and telophase. No significant difference in this behaviour could be noted between seeds germinated after pre-soaking and without pre-soaking.
4. Gradual degeneration of fragments with increase in age, occurrence of micronuclei and the fate of these fragments and nuclei have been studied

and their significance discussed.

5. Data have been gathered to show that possibly the secondary constriction regions are involved in breakage. The susceptibility of secondary constriction regions to breakage has also been regarded as an indication of its chemical difference from the rest of the chromosome segments.

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