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The Effect of Caffeine on Pisum sativum with Emphasis on Meiosis

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The Effect of Caffeine on *Pisum sativum* with Emphasis on Meiosis

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INTRODUCTION AND LITERATURE SURVEY

In the previous studies on the effects of caffeine, only the immediate mitotic effects on root tips were investigated. The main purpose of the present study was to investigate the long term meiotic effects of caffeine on Pisum sativum.

In specific concentrations, caffeine has been reported to behave as a mutagenetic compound in the following plants: Ophiostoma multivannulatum (ascomycete), Escherichia coli (bacterium), Allium cepa (onion), Vicia faba (broad bean), and Pisum sativum (pea) (Fries and Kihlman, 1949; Kihlman and Levan, 1949, 1951; Kihlman, 1952; Gezelius and Fries, 1952; and Zetterberg, 1960).

Generally, concentration levels of 1.0 to 2.0% are considered lethal. While twenty-four hours of treatment with 0.4% caffeine produces total suppression of mitosis, lower concentrations have only a suppressive action on the frequency of mitosis. Concentrations of 0.02% and lower allow normal number of mitosis or even an increase in the rate of mitosis (Kihlman and Levan, 1949).

Specifically, the reported mutagenetic effects can be broken down into three categories: (1) Production of C-mitosis, (2) suppression of cell wall formation, and (3) primary and secondary changes in

to chromosomes brought about by mitotic delay (Kihlman and Levan, 1949).

C-mitosis is defined as an inactivation of the spindle apparatus connected with a delay of division of the centromeres (Levan, 1949). The result is that the chromosomes of full c-metaphase are scattered over the entire cell (Kihlman, 1966).

The suppression of cell wall formation leads to binucleate cells, and later to multinucleate cells. The threshold for this reaction lies between 0.02% to 0.04% concentrations of caffeine. At 0.04% cell wall formation is often checked, but the reaction is not complete. This reaction causes cells with high numbers of chromosomes to arise. These nuclei often fuse during mitosis, giving rise to large clusters of chromosomes. Sometimes the two nuclei of one binucleate cell are arranged in tandem, and during mitosis result in four anaphase nuclei. The nuclei in the middle are pushed together from both sides and form one telophase nucleus. The result will be one diploid nucleus at each end of the cell, with one large tetraploid nucleus in between. After prolonged treatment, the exposed tissue will have small diploid cells mixed in with different kinds of polyploid cells, causing a highly unorganized mass of tissue. In such tissue, variation in the direction of the spindle axis occurs and frequently does not correspond with the direction of the root axis (Kihlman, 1949, 1966).

Primary (physiological) and secondary (structural) changes

occur after treatment with caffeine. The primary effect is induced in dividing cells and is characterized by stickiness and pseudo-chiasmata. Physiological changes are usually seen directly after the start of the treatment. Most of these changes are due to the stickiness of the chromosome matrix, causing the clumping of the chromosomes at metaphase. The most common change due to this stickiness is the formation of the false anaphase bridges. This difficulty of separation of chromosomes usually affects only one or a few chromosomes per cell (Kihlman and Levan, 1949). However the persistance of these false bridges may result in breaking and refusing of individual chromosomes.

The secondary or structural changes that occur are observed in cells entering mitosis at the end of the mitosis-free period, which follows treatment. These effects are thus induced in resting cells. The changes that were observed were of the usual type found in cells after X-ray and mustard gas treatment. These chromosome changes are known as radiomimetic changes and they include fragmentations and chromosome bridges at anaphase. These bridges expose most of the dicentric chromotids which have arisen as a result of sister chromatid reunion in centric fragmentations at the point of breakage, or as a result of reciprocal translocation (Kihlman, 1952b).

Since the morphological type of these bridges is often indistinguishable from that of sticky bridges, the separation of these two types is difficult. Kihlman and Levan (1948) transferred caffeine

treated onion bulbs to pure water after 24 hours treatment. After 6-12 hours in water, physiological changes ceased to occur. The remaining bridges were then due to irreversible structural changes.

Other purine derivatives, especially 8-substituted caffeine derivatives, produce a very strong secondary effect. Some chromosomes were found to be so fragmented that no anaphase movement was possible. Ring fragments were frequently apparent. Micronuclei were formed in interphase by those acentric fragments which, during mitosis, were not included in either of the daughter nuclei. Sister chromatid union at the point of breakage in centric as well as in acentric fragments were common as mitotic translocation (Kihlman, 1951).

Chemically, caffeine is a methylated oxypurine. There are two types of these methylated oxypurines. One group, of which 8-ethoxycaffeine (EOC) is the representative type, consists of the 8-ethers and 8-tioethers of caffeine. The effects of these oxypurines are independent of the mitotic activity during treatment. The other group, which 1, 3, 7, 9-tetramethyluric acid (TMU) is a representative type, includes caffeine. The effects of these compounds is dependent on the mitotic activity during the treatment period. One explanation of this difference is attributed to a difference in ability to penetrate the nuclear membrane at interphase. Purines of the EOC type are able to penetrate the nuclear membrane, but the TMU type can only enter the cell during cell division, when the nuclear membrane is dissolved (Kihlman, 1961).

If the difference in the mode of action between the TMU and EOC type of purine derivatives is really a difference in penetration ability, then the Lipoid Theory of Overton seems valid. Overton observed that compounds with higher solubility in fat solvents were more capable of penetrating the living cell membrane. According to this theory, it is then the relative lipoid solubility or the distribution in the lipoid/water system of an organic compound, which is decisive in the penetration ability (Overton, 1899).

EOC purines of the TMU type, including caffeine, have a stronger affinity for the water phase. The presence of a perinuclear lipoidic layer in the nuclear envelope of root tip cells of onion was determined (Kihlman, 1951). This would explain the significance of relative lipoid solubility of purine derivatives in their ability to penetrate the nuclear envelope.

A further explanation for the differences in chromosomal effects of the TMU and EOC types is that they are absorbed differently inside the nucleus (Kihlman, 1961). The purine derivatives with surface activity, after entrance into the nucleus, probably accumulate on the intra-nuclear surfaces of the nuclear membrane. The purines would first exceed their threshold concentrations for chromosomal breaking activity on these surfaces. Those chromosomes in contact with these surfaces would be the first to be broken (Kihlman, 1952a).

Regarding radiomimetic activity in general, a surface for

boundary activity may be significant, if the purine derivatives must be absorbed on or come in contact with the chromosome before the breaks can be induced. Purine derivatives that are very active inducers of chromosome breaks also have a high boundary activity and those purine derivatives which are inactive or only slightly active were found to have a low boundary activity in general (Kihlman, 1952b).

A connection has been found between molecular structure and activity. Factors of importance are degree and type of N-alkylation, number and type of carbon-bound electronegative substituents, and the number and position of the double bonds. Activity is not bound to any particular atom or group of atoms, but is dependent on the molecule as a whole (Kihlman, 1951).

There has been found a good correlation between the solvent power and the radiomemetic activity of purines. The solvent power of purines is dependent on the same intermolecular attraction forces which are responsible for the formation of molecular compounds (Weil-Malherbe, 1946). It is concluded that these "intermolecular attraction forces" also play a significant role in the radiomimetic activity of purines. This does not necessarily mean that the purines act directly on the intermolecular bonds in chromosomes. Chemically, purines are very inactive, so it would be improbable that purines would cause chromosome breaks directly by reacting with some part of the chromosome. A competitive inhibition of the enzymes is probably dependent

upon molecular attraction between enzyme proteins and inhibitor substances (Kihlman, 1951, 1952b).

MORPHOLOGY OF THE CHROMOSOMES OF PISUM SATIVUM

The seven chromosomes of Pisum sativum are of about equal size. The longest is about 6u, and the smallest about 4u. According to Kihlman (1952a) the chromosomes are described as follows and diagrammed in Figure 1.

I. The largest chromosome has a submedian centromere. Sometimes a secondary constriction is apparent on the longer as well as on the shorter chromosome arm..

II. Comparatively small chromosome with a practically median centromere and no secondary constriction.

III. The same size as II. The centromere is practically median. A subterminal secondary constriction separates a segment having a narrower diameter than the rest of the chromosome.

IV. The second largest chromosome. Centromere is subterminal. A subterminal secondary constriction separates a segment of the same diameter as the rest of the chromosome, from the longer chromosome arm.

V. Somewhat shorter than IV. Centromere is practically subterminal. Secondary constrictions lacking.

VI. The satellite chromosome. The same size as V. The

centromere is subterminal. The longer arm carries the satellite, which has a smaller diameter than the chromosome body. VII. Same size as V and VI. This chromosome has the most terminal centromere, the length of the shorter arm being only one fourth that of the larger arm. Probably no secondary constriction.

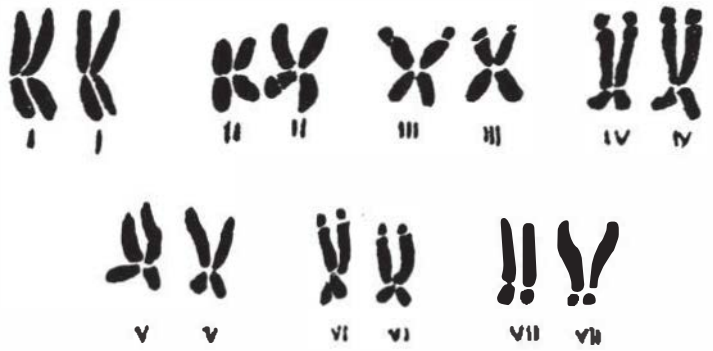

Figure 1. Chromosomes of Pisum sativum (Kihlman, 1952a).

MATERIALS AND METHODS

The variety of pea used in this experiment was the Little Marvel Improved. These peas were allowed to soak in concentrations of caffeine and time intervals that have been used by previous investigators. The concentrations were: 0.2%, 0.15%, 0.10%, 0.05%, and 0.025%. The lengths of time used were: 5 min., 10 min., 15 min., 30 min., 45 min., 60 min., and 12 hours. These time intervals were used for each caffeine concentration.

After the peas were soaked for the prescribed length of time they were planted in pots and allowed to germinate. A set of controls were also planted. Pictures were taken using a Pentax 135 mm camera and Kodak Plus X Panchromatic film, of the pea plants after two weeks and after twelve weeks of growth. The first flower buds began to appear after four weeks.

The immature buds were harvested and fixed in a solution of one part glacial acetic acid and three parts 95% ethyl alcohol for 12 to 24 hours. The buds were then removed to a solution of 70% ethyl alcohol. The more mature buds were harvested and retained for analysis of the normality of the pollen grains. Before the plants were discarded a final height was recorded for each plant.

The anthers were dissected out of the immature buds that were fixed and preserved. Anther smears were made using iron-aceto carmine as the chromosome stain. These temporary chromosome preparations were photographed using a Nikon microscope and camera. The film used was Kodak Plus X Panchromatic.

The pollen grains were stained in methyl blue in lactophenol. This stains the protoplasm of the pollen grain a dark blue if the pollen grains are normal. If they are abnormal the cell will not take up the stain as well, because the protoplasm will have shrunken away from the cell wall (see Figure 2).

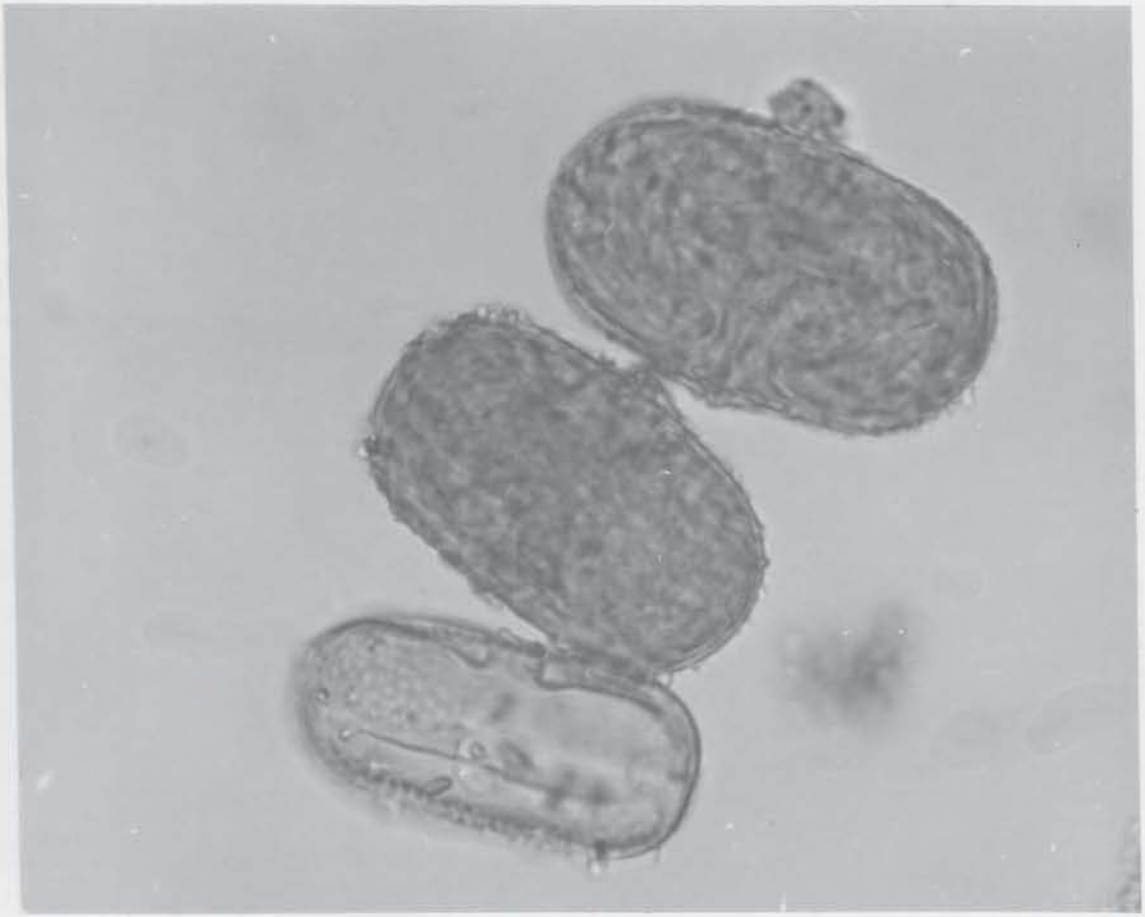

Figure 2. Pollen grains formed after treatment of seeds of Pisum sativum with 0.10% caffeine for 12 hours.

RESULTS AND DISCUSSION--CYTOLOGY

Observations of meiotic configurations were made from pollen spore mother cells undergoing meiosis. A comparative analysis was made of different meiotic phases between pea plants of different caffeine concentrations and different lengths of time and meiotic phases in the control plants.

Meiotic phases that were examined in particular were diakinesis, metaphase I and II, and anaphase I and II. The meiotic cells were examined for chromosome changes such as stickiness of chromosomes, anaphase inversion bridges, chromosome fragments and occurrence of polyploid cells. All of these changes occurred in previous studies on the immediate effects of caffeine on plant roots during mitosis.

Inhibition of cell plate formation causing polyploid cells has been attributed to caffeine (Kihlman, 1949, 1966; and Kihlman and Levan, 1949). Stickiness of chromosomes was reported by Kihlman and Levan (1949) and occurrence of fragments and anaphase bridges was reported by Kihlman (1952b).

In the present study's comparative analysis of meiotic configurations neither anaphase inversion bridges or chromosome fragments were found to occur. Polyploid cells, which would have indicated

failure of cell wall formation, were not detected.

With regards to stickiness of chromosomes, there were two concentrations and time levels that exhibited some degree of abnormality. Figure 3 illustrates early diakinesis at 0.025% and 60 minutes. At this point, no chromosomal abnormalities can be observed. However during early telophase I, two percent of the pollen mother cells showed late disjunction of the homologs during anaphase I (Figure 4).

Time and concentration levels at 0.2% and 60 minutes, also had no effect during daikinesis and metaphase I (Figures 5 and 6). During anaphase I, several pollen mother cells illustrated two or more chromosome pairs being separated late (Figures 7 and 8).

Neither of these two types of abnormal chromosomal behavior seemed to have an effect of the percentage of fertility of the mature pollen grains (Figure 2) since very few abortive pollen grains were observed.

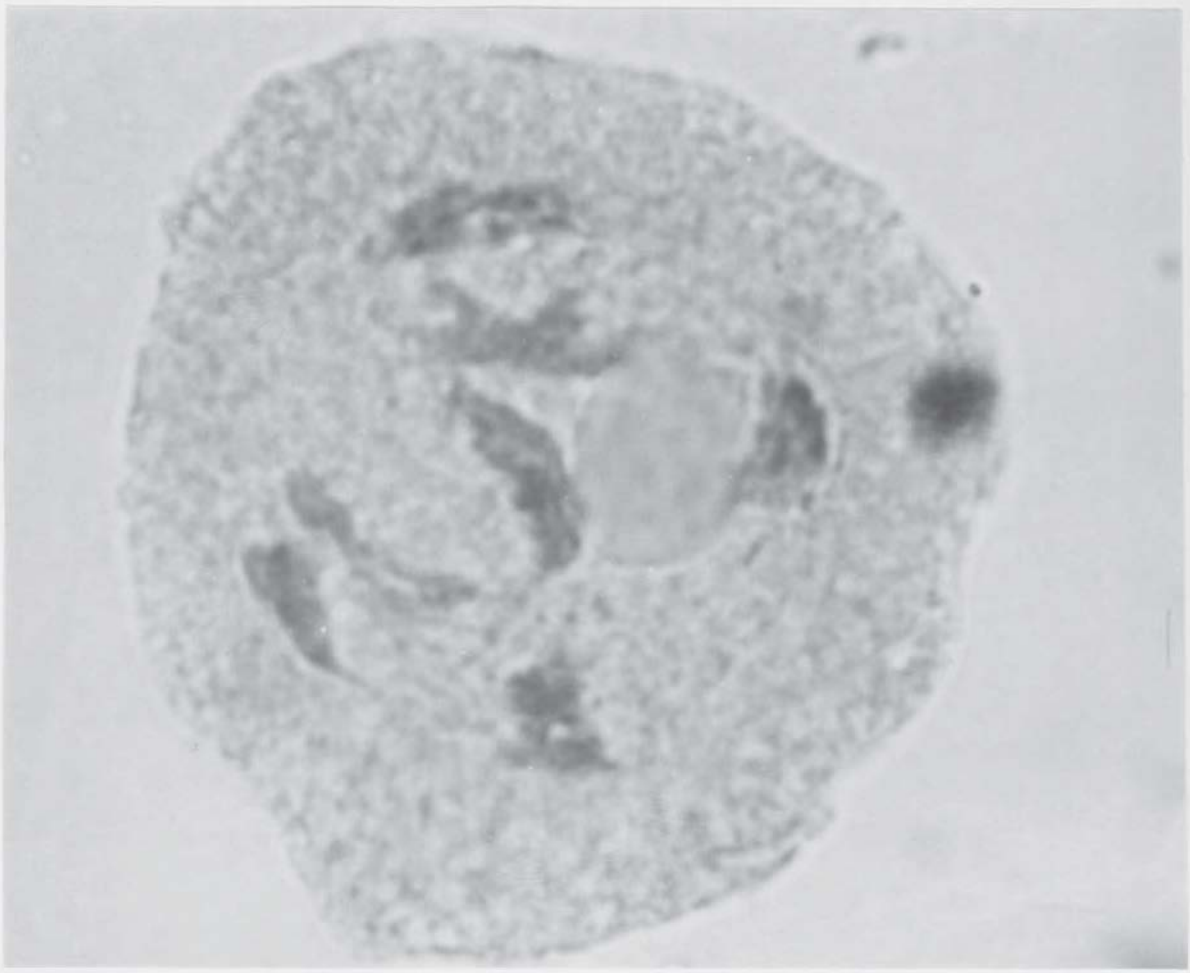

Figure 3. Early diakinesis in microspore mother cells after treatment with 0.025% caffeine for 60 minutes.

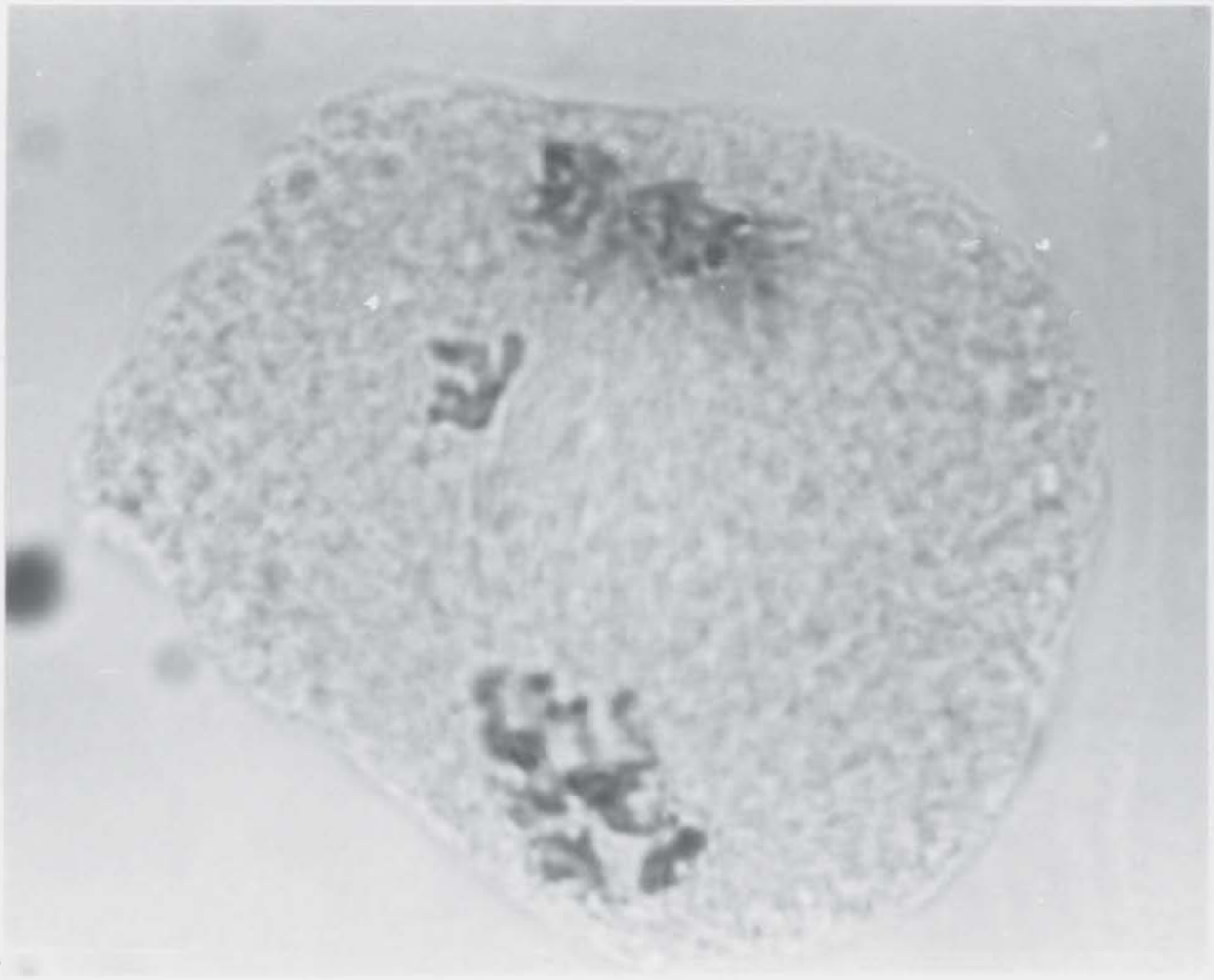

Figure 4. Telaphase I illustrating late disjunction of homologs after treatment with 0.025% caffeine for 60 minutes.

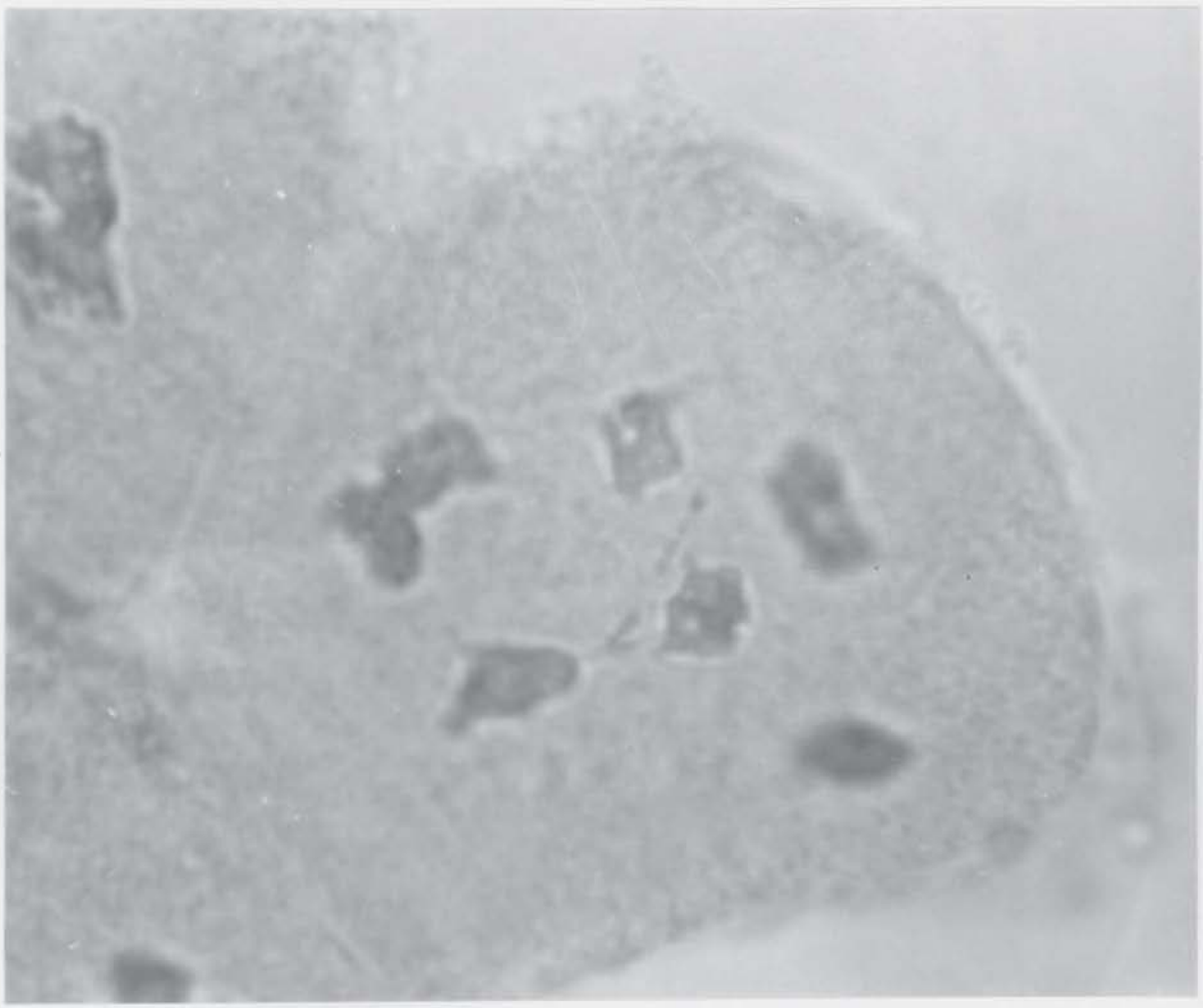

Figure 5. Diakinesis after treatment with 0.2% caffeine for 60 minutes.

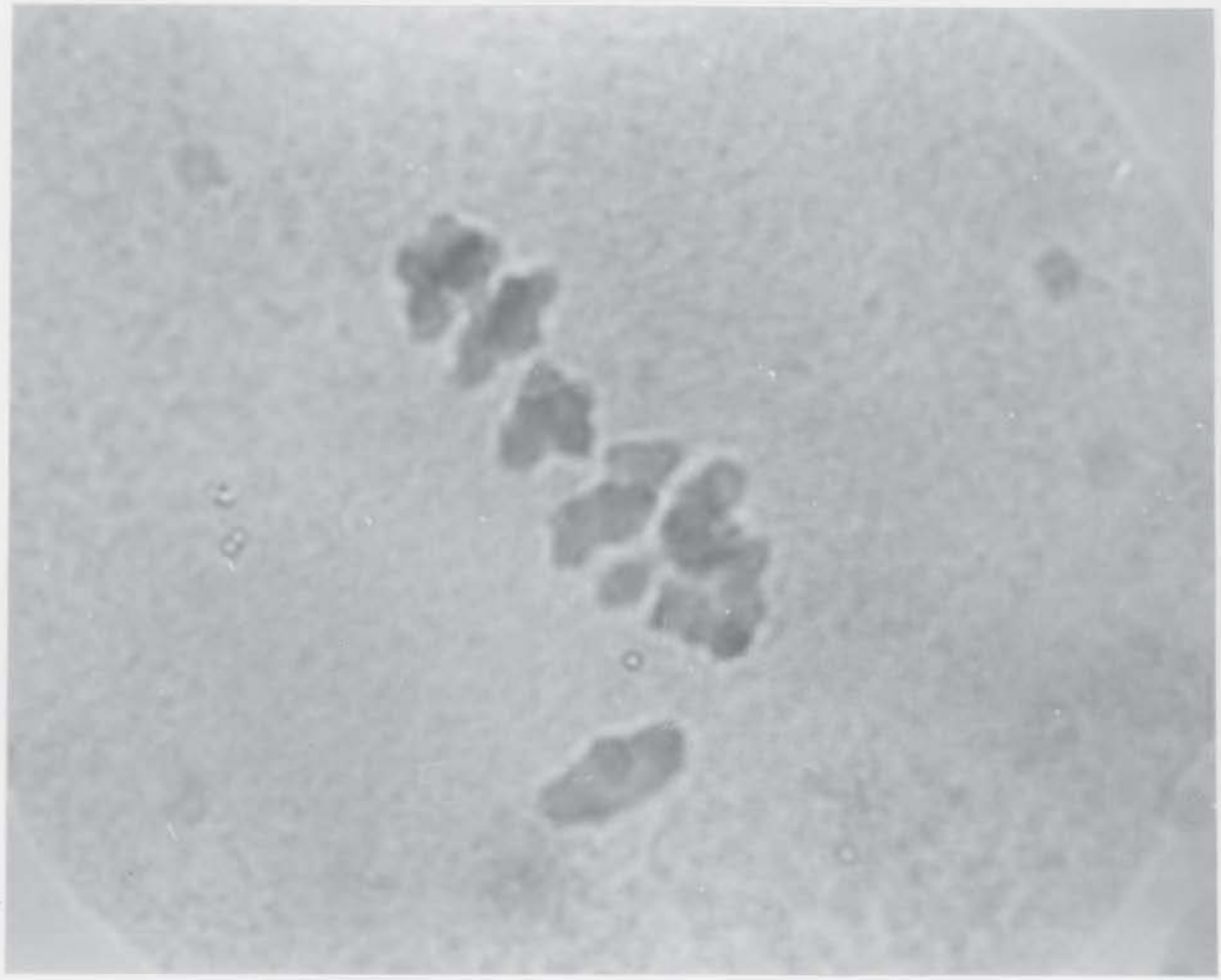

Figure 6. Metaphase I after treatment with 0.2% caffeine for 60 minutes.

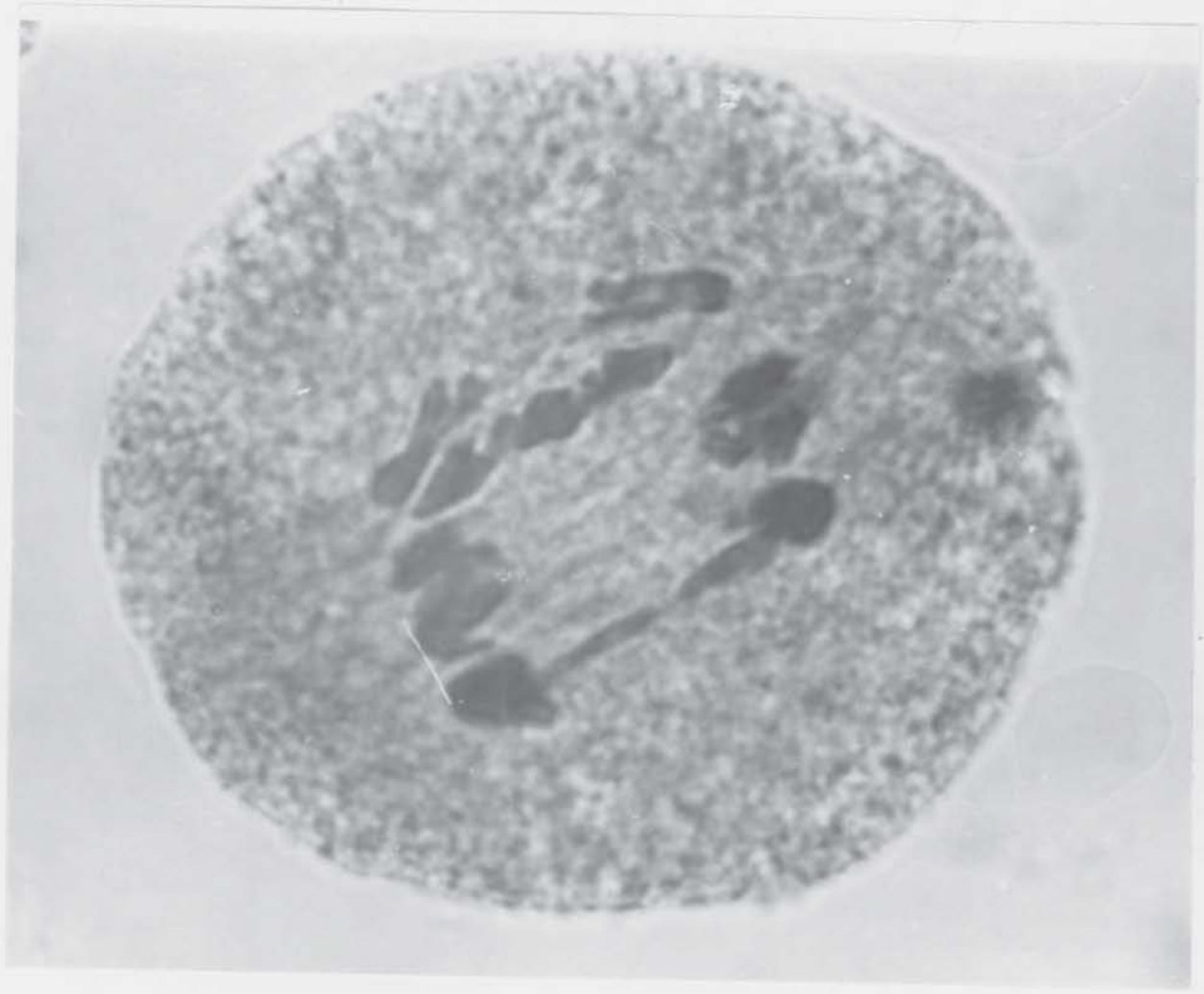

Figure 7. Anaphase I after treatment with 0.2% caffeine for 60 minutes, illustrating late separation of chromosomes.

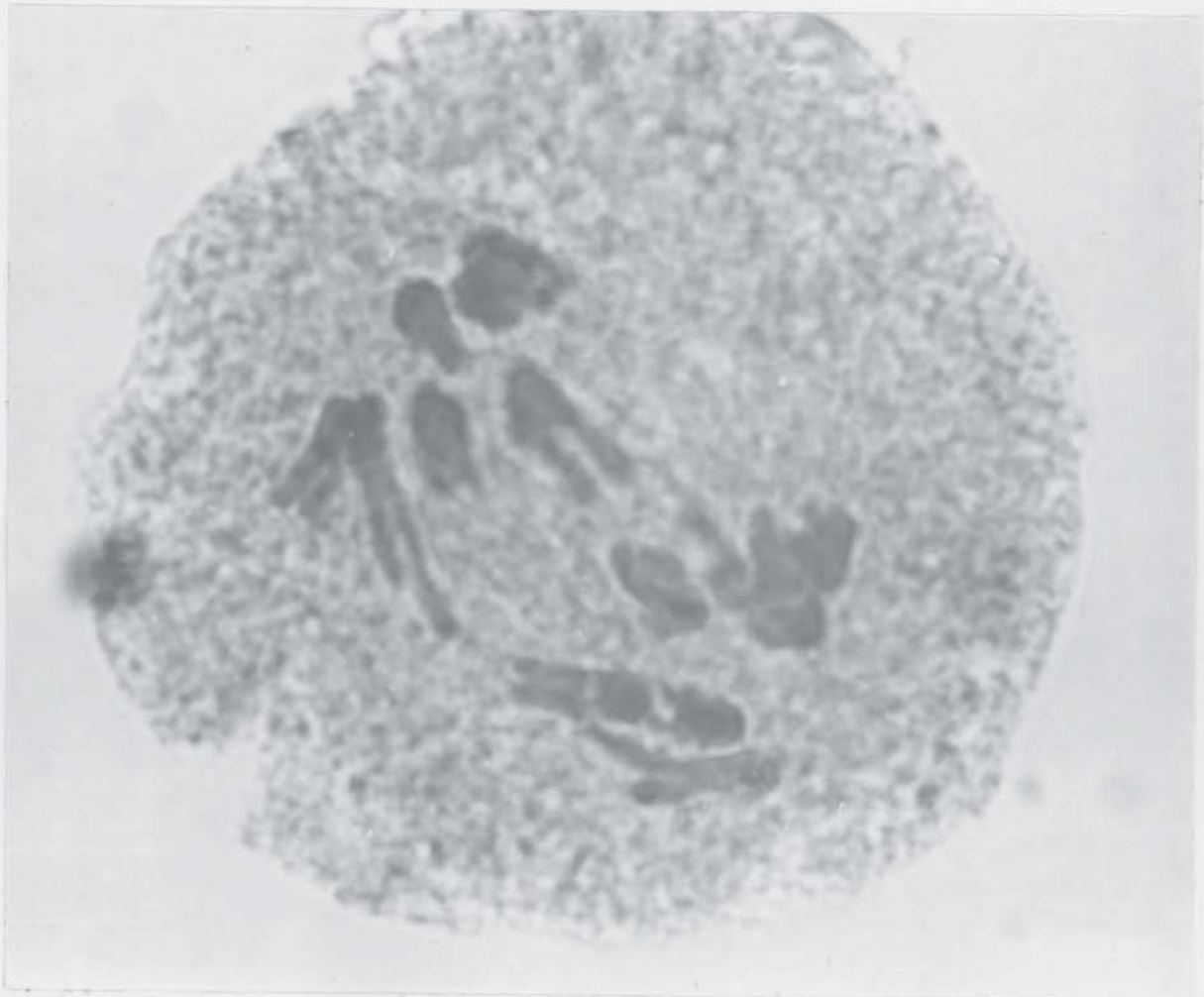

Figure 8. Anaphase I after treatment with 0.2% caffeine for 60 minutes, illustrating late separation of chromosomes.

RESULTS AND DISCUSSION--PHENOLOGY

Comparisons of seed germination and height attained by plants were made and presented in Tables I and II.

TABLE I

GERMINATION RATE
(Number of seeds germinated out of 5 planted)

Time in minutes	Concentrations of Caffeine in percent					
	0.2	0.15	0.1	0.05	0.025	Avg. %
5	5	3	5	4	4	84
10	2	3	5	4	5	76
15	3	4	5	4	5	84
30	3	3	5	3	5	76
45	4	2	5	2	5	72
60	4	3	5	4	5	84
720	3	3	2	3	3	56
Avg. %	68	63	91	68	91	

Control - 7 out of 10 - 70%

From the above table it can be noted that after seeds were in caffeine for twelve hours, only 56% of these seed germinated. This seems to indicate that prolonged treatment in caffeine might reduce viability of seeds.

TABLE II

HEIGHT
(Average height in mm)

Time in minutes	Concentrations of Caffeine in percent				
	0.2	0.15	0.1	0.05	0.025
5	28.4	34.3	48	20	23.2
10	26	23	23.7	35.5	28.8
15	29.7	23.8	24.6	24.3	16
30	30	25.7	23.2	22	22.3
45	23.5	20	19.3	24	19.3
60	45.3	25.3	16.8	22	23
720	16.3	22	23	20.8	22.3

Control average height - 34.8

Since peas are self-pollinators and the seeds were all of one variety, it would be expected that the range of heights would be fairly uniform. In both the controls and the experimental plants, however, there were a few exceptionally tall individuals. This is illustrated in the controls in Figures 9 and 10, and at 0.20% concentration at 60 minutes in Figures 17 and 18. This range of heights cannot be due to the caffeine since the controls also exhibited this range.

CONTROL

Figure 9. After 2 weeks growth.

Figure 10. After 12 weeks growth.

Caffeine Concentration of
0.025% at 60 min.

Figure 11. After 2 weeks growth.

Figure 12. After 12 weeks growth.

Caffeine Concentration of
0.15% at 15 min.

Figure 13. After 2 weeks growth.

Figure 14. After 12 weeks growth.

Caffeine Concentration of
0.15% at 45 min.

Figure 15. After 2 weeks growth.

Figure 16. After 12 weeks growth.

Caffeine Concentration of
0. 2% at 60 min.

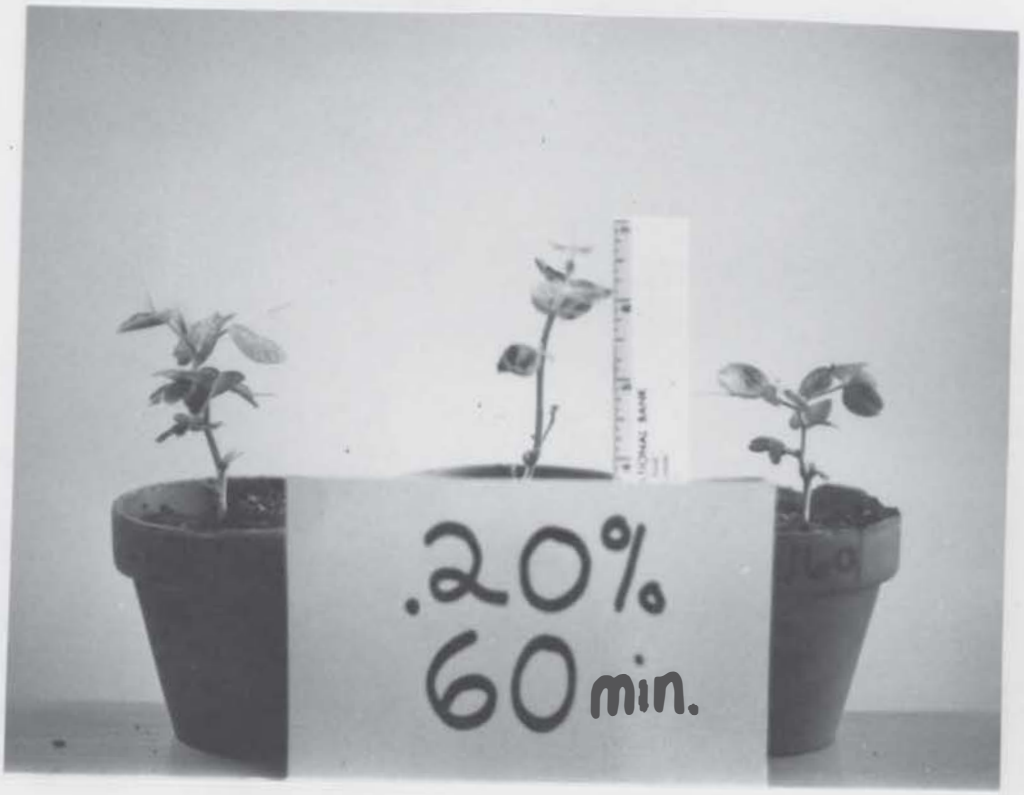

Figure 17. After 2 weeks growth.

Figure 18. After 12 weeks growth.

CONCLUSIONS

The only abnormalities that were observed were a few cases of stickiness of chromosomes during anaphase I separation of homologous chromosomes. This stickiness of chromosomes that was observed at meiosis would not be the same type of stickiness as reported by Kihlman (1949) in his study of root tips. His observations were due directly to the effect of caffeine, probably due to interruption of enzymatic activity. Caffeine was no longer present when the plants were undergoing meiosis in this study. Stickiness during meiosis in the present study would be due to a residual effect of caffeine. A change that caused an increase in production of the histones of the histone matrix of the chromosomes could cause stickiness.

The concentration level and amount of caffeine that was actually inbided through the seed coat and cell membrane of the actively dividing cells of the embryo was not determined. This is important, since caffeine cannot penetrate the nuclear membrane, therefore the cells must be actively dividing for caffeine to have an effect on chromosomes. Caution must be taken in drawing inferences from negative results obtained on nondividing cells (Kihlman, 1951). So this lack of changes was not necessarily due to the fact that caffeine does not

cause long term effects in plants, but because there might not have been enough caffeine present when active cell division was taking place, to cause more permanent changes.

Modifications that should be made in subsequent studies of the long term effects of caffeine on plants, would include germination of the seeds prior to treatment with caffeine. This would insure that active cell division was taking place during treatment.

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