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A COMPARATIVE STUDY OF TEMPERATURE-INDUCED DIFFERENTIAL STAINING PATTERNS IN *TRILLIUM SESSILE* L.

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ABSTRACT

Combinations of certain commonly used stains and fixatives were employed in studies of temperature-induced differential staining patterns of population samples of *Trillium sessile L.* from Nashville, Tennessee. The plants were cold treated and the temperature induced differential staining of their chromosomes were compared after preparation with several staining and fixation procedures. The fixatives employed in making the smear preparations were LaCours 2BE and three variations of Carnoy's fixative. The stains utilized were acetocarmine, propionic carmine, and the Feulgen stain. Overall chromosome length and heterochromatic segment lengths of each type of chromosome prepared with each stain and with each fixative were recorded and compared by analysis of variance. From the statistical analyses, it was shown that there is no alteration in the length of the heterochromatic bands or in the chromosome itself due to the procedure utilized in preparation.

INTRODUCTION

The genus *Trillium* L. (Liliaceae, Tribe Paridae) has been the object of numerous cytological investigations. The frequent use of *Trillium* plants has apparently resulted from the small number ($x = 5$, $2n = 10$), large size, and morphological distinctiveness of their chromosomes. An additional feature, which has contributed to much of the cytological interest in *Trillium*, is the phenomenon of temperature-induced differential chromosome staining. When meiotic or mitotic materials are treated at 0 ± 2 C for approximately 72-96 hours, certain regions of the chromosome partially lose their ability to react with nuclear stains. The resulting understained regions are referred to as heterochromatic segments, H-segments, or simply as heterochomatin (Darlington and LaCour, 1938). The nature of the heterochromatic segments and the reasons for the differential reactivity to cold treatment have been the object of numerous investigations (Darlington and LaCour, 1938, 1940, 1941; Lima-De-Faria, 1959; Boothroyd and Lima-De-Faria, 1964; Woodard, *et al.*, 1966; Caspersson, *et al.*, 1969). However, more investigation is needed before the chemical nature and structure of the H-segments are clarified.

Many workers have adopted the use of temperature-induced differential staining patterns as characters of systematic value (Haga, 1937, 1953, 1956; Haga and Kurabayashi, 1953; Bailey, 1952, 1958; Kozuka and Kurabayashi, 1959; Samejima and Samejima, 1962; Kurabayashi, 1963; Serota, 1967, 1969). In these investigations, however, there has been very little uniformity with regard to the fixation and staining procedures used, various investigators having used different types of stains and fixatives in obtaining differential reactivity.

The present work is a comparative study concerning effects of selected fixatives and stains upon a standard type of temperature-induced staining pattern to determine whether or not these various staining and fixation procedures produce comparable results.

PRELIMINARY CONSIDERATIONS

Within members of genus *Trillium,* meiosis occurs either during the winter or in early spring, when the plants are extremely small and the flowers are still in the bud stage. Therefore, meiotic material could not be utilized in the present study. Root tips of *Trillium* provide excellent mitotic material for cytological analysis; however, the number of root tips per rhizome is small, and they are easily broken when the plants are collected. Another source of mitotic tissue is ovules. This offers the advantage of providing large quantities of mitotic cells, so that identical plant material can be exposed to different types of fixation and staining procedures. Also, plants can be collected in the field and transplanted to an experimental garden or to a greenhouse without damage to the ovules. In view of these advantages, ovule tissue was selected for use in the present work.

The stains and fixatives chosen for this study were limited to those most frequently utilized in previous investigations. The stains, acetocarmine, propionic carmine, and Feulgen (leuco-basic fuchsin), and the fixatives, LaCour's 2BE and three variations of Carnoy's fixative, were employed most frequently by previous workers (Belling, 1921; Darlington and LaCour, 1947; Sparrow and Sparrow, 1949; Serota, 1967).

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In comparing effects of different stains and fixatives upon chromosomal staining patterns, there is always the possibility of variation between and among individuals within the population. The only way completely to avoid these variables is to subject genetically identical material to the various procedures. Ideally, therefore, one would use materials from the same plant and simultaneously subject these identical samples to the various combinations of fixatives and stains. However, the small number of ovules of each *Trillium* plant prevents this approach from being used.

Nevertheless, sufficient ovule material is available in each plant to provide for some limited degree of comparison with genetically identical material. This is to say that enough ovules are produced by the flower of each plant to permit variations of either fixation or staining procedures, but not both in their various combinations. Since four different fixatives were being utilized in the study along with only three stains, it was decided to vary the fixation procedure while keeping the staining technique constant. Using this approach, each ovary could be divided into four approximately equal parts, and each of these quarters could be fixed with a different fixative. By staining these four kinds of fixed material by the same technique, all factors remain constant except the fixative and fixation procedure used.

Materials and Methods

Plants of *Trillium sessile* L. were obtained from a population located in Nashville, Tennessee, on the west side of Tyne Boulevard, one mile east of the intersection of Tyne Boulevard and Hillsboro Road (U.S. Hwy. 431). Plants were collected and transferred to flats in the greenhouse at George Peabody College. The plants were subjected to cold treatment in sets of 15 individuals. Whole plants were placed in plastic bags along with moist peat moss, the bags tightly sealed, and treated at 0 ± 2 C for 96 hours. The plants were then removed and the ovary carefully excised, cut into quarters, and subjected to fixation.

Each quarter of the sectioned ovary from each plant was placed in a separate small glass vial, to which was added approximately 5 ml of one of the fixatives selected for use. The first quarter of the ovary was fixed in LaCour's 2BE, the second quarter in Carnoy's I, the third quarter in Carnoy's II, and the last quarter in Carnoy's III fixatives (Table 1). All fixatives were precooled and fixation carried out at 5°C, to prevent any warming of the cold treated plant materials during the early stages of fixation. Materials were fixed in LaCour's 2BE for 15 minutes, washed with water and then stained immediately, whereas those materials fixed in the various types of Carnoy's were fixed for 24 hours before being stained.

Table 1. Chemical Composition of Fixatives Most Commonly Used In Cytological Studies of *Trillium*.

LaCour's 2BE: according to Darlington and LaCour (1947)		
chromic acid	90.0	ml
potassium bichromate	1.0	g
saponin	0.05	g
acetic acid (5%)	10.0	ml
osmic acid (2%)	15.0	ml
distilled water	45.0	ml
Carnoy's I: according to Serota (1967)		
absolute ethanol	3	parts
propionic acid	1	part
chloroform	1	part
Carnoy's II: according to Warmke (1935)		
absolute ethanol	3	parts
glacial acetic acid	1	part
Carnoy's III: according to Darlington and LaCour (1947)		
absolute ethanol	6	parts
chloroform	3	parts
glacial acetic acid	1	part

Upon completion of fixation, the ovary sections were stained by one of three staining procedures (Table 2). One set of 15 plants (60 ovary sections) was stained with acetocarmine according to the methods of Belling (1926) and Serota (1967). A second set of 15 plants was stained with propionic carmine as described by Sparrow and Sparrow (1949). The third set of plants was stained by the Feulgen reaction, as described by Darlington and LaCour (1947), and Matsuura, *et al.* (1962) in their work with *Trillium*.

Table 2. Composition of Nuclear Stains Most Frequently Employed In Cytological Studies of *Trillium*.

Acetocarmine		
glacial acetic acid	45.0	ml
distilled water	55.0	ml
carmine	0.5	g
Propionic Carmine		
propionic acid	45.0	ml
distilled water	55.0	ml
carmine	0.5	g
Feulgen (leuco basic fuchsin)		
distilled water	100.0	ml
basic fuchsin	0.5	g
sodium bisulfite	0.5	g
1 N hydrochloric acid	10.0	ml

Before staining with acetocarmine or propionic carmine, the fixed ovaries were washed in 70% ethanol. The ethanol was decanted, a few drops of stain added to the vial, and the material stained for approximately 5 minutes. Ovules were then teased out of the ovary and mounted in a drop of the stain on a clean microscope slide. Temporary smear preparations were made according to standard squash techniques.

The Feulgen reaction entails a more complicated procedure than that used for the acetocarmine or propionic carmine stains. After fixation, the ovary sections were washed in cold running tap water for 15 minutes. Approximately 5 ml of 1 N HC1 was then introduced into the vials, and the sections hydrolyzed for 15 minutes at 60°C. The HC1 was discarded, and the ovary sections washed again for 15 minutes. The Feulgen reagent was then added and the tissue stained for a period of two hours. A final tap water wash for 10 minutes completed the process, and the ovary sections were stored in 45% acetic acid to await squashing. Ovules were squashed using 45% acetic acid as the mounting medium and the slides prepared as before.

Slides were observed using a Wild compound light microscope. Camera lucida drawings and photomicrographs were made of representative chromosome spreads produced by the various combinations of stains and fixatives. The five morphologically distinct chromosomes of the haploid genome were lettered A — E using the Japanese system (Fig. 1). The quality of the preparations according to the stain and fixative used was also noted.

In scoring results, measurements of a comparative nature were recorded to determine the effects of the various stains and fixatives upon chromosome size as well as upon H-segment size. To detect overall changes in chromosome lengths resulting from the different procedures used, the total lengths (long arm plus short arm) of randomly selected chromosomes of each of the five types were scored. Twenty measurements of the lengths of each lettered chromosome prepared by each combination of each stain and fixative were recorded. All measurements were taken at a total magnification of 1,000 X using a calibrated ocular micrometer.

In scoring the results, a set number of measurements was taken of chromosomes of each lettered type, with each stain, and with each fixative. Thus, three variables were considered: 1) the designated chromosome — A, B, C, D, E; 2) the stain employed — acetocarmine, propionic carmine, or Feulgen; and 3) the fixative used — LaCour's or one of the three Carnoy's fixatives. Analysis of variance was selected as a means of examining the scored data because it is a technique for determining if significant difference exists among more than two groups of means, and it permits the investigation of one set of variables in combination with another set or sets.

The comparative lengths of the observed heterochromatic segments were used as an indication of the procedure employed. For simplification in scoring, all measurements of length were recorded in units of a calibrated micrometer (one unit being equal to 1.42857 x 10–3mm). All calculations were made in terms of these measurement units.

Before any differences in the H-segments could be investigated, it had to be shown that the total chromosome length was not altered significantly by the preparation procedure followed. Therefore, a preliminary analysis of variance of the total lengths of all the lettered types of chromosomes with each fixative and stain combination was undertaken (Table 3). This analysis is of a 5x4x5 factorial design.

To determine statistically the effect of the stain or fixative on the length or size of the H-segments involved, analysis of variance tables of a completely random design were calculated for each heterochromatic segment in each individual chromosome. Four of the chromosomes displayed H-bands, two of them containing more than one band (cf. Fig. 1). Since the A chromosome had no heterochromatic segment, it will not be discussed.

Results

From the F values listed in Table 3, it is evident that the effects due to interactions of the variables (stain, fixative, and chromosome type) are not significant. The importance of the main effects is also noted, and two of these effects are found to be non-significant. The effect of the fixative alone and the effect of the stain alone are not responsible for any variation in total chromosome length, although, the type of chromosomes considered is found to be of significance. This is to be expected, however, for there are five morphologically distinct chromosomes in the set, each with a specific average length. It is concluded that the total chromo some length is not altered significantly as a result of the effect of the stain or fixative independently or in combination.

The B chromosome had one heterochromatic segment in the long arm that was 0.82 ± 0.046 units in length. Twenty measurements of this particular H-segment, and of all the other H-segments in each of the other three chromosomes, were taken with acetocarmine and propionic carmine stains. Ten measurements were taken with the Feulgen stain because of a limited supply of material; however, analysis of variance also allows for differences in the sizes of the groups being compared. The F values showed no significant effect in the length of the chromosome due to stain. Since there was no significant alteration in the general size of the chromosome due to stain-fixative interactions, none was expected in the H-segment size. Therefore, it was concluded that there was no significant change in the length of the H-band in the chromosome due to the stains and fixatives employed.

Table 3. Analysis of Variance Table (5x4x5 Factorial Design) Total Chromosome Length in All Stain-Fixative Combinations.

	df	SS	MS	F
A=stain	4	808.346	201.825	.42
B=fixative	3	932.883	310.961	.64
C=lettered	4	13,943.596	3485.899	7.17**
Type of Chromosome				
AB	12	419.182	34.932	.07
AB	16	465.884	29.680	.06
BC	12	610.972	50.914	.10
ABC	48	235.588	4.908	.01
Replications	9	475.961	52.884	.11
Error	891	433,047.588	486.024	
Total	999	450,879.000		

**Significant at the 1% level

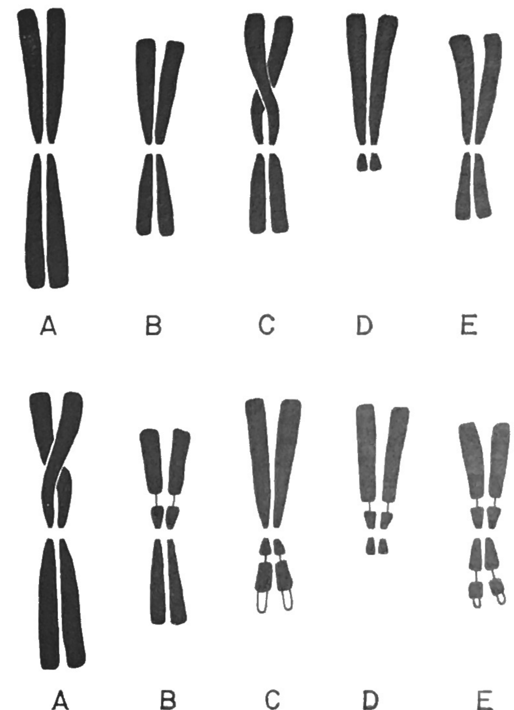

Figure 1. Idiograms showing the basic haploid genome of *Trillium*, along with the differential staining pattern observed in the population of *Trillium sessile* used in this investigation.

The C chromosome had two H-segments, both in the short arm. One heterochromatic segment was terminal in position (3.59 ± 0.142 units in length) while the other band was subterminal in position (0.84 ± 0.046 units in length). Analysis of variance tables for both the terminal and subterminal segments showed all F values to be non-significant. Therefore, there was no

alteration shown in either the terminal or subterminal H-bands of the C chromosome due to the stain or fixative used.

The D chromosome displayed only one H-segment located near the kinetochore in the long arm. Analysis of variance again indicated no significant variation in its size. The average length of the segment was 0.74 ± 0.041 units.

The E chromosome had the most complex differential staining pattern. It contained three H-bands, two in the short arm and one in the long arm. The terminal H-segment in the short arm (1.00 units in length) was very consistent in length. Since the variation of overall chromosome size was insignificant (cf., Table 3) so was the variation in the size of this H-segment. The interstitial H-band of the short arm averaged 1.03 ± 0.046 units in length. The F values were non-significant, indicating no alteration in this segment resulting from the procedure used. No significant variation in size due to the stain or fixative employed was displayed by the final heterochromatic segment in this chromosome. The average length of this segment was 0.99 ± 0.049 units.

Although the statistical data show that the stains and fixatives employed in this study have no effect on the differential staining pattern of the chromosomes, the quality of the smear preparations did vary, in some instances, according to the procedure utilized. The Feulgen reaction produced smears of good quality and was satisfactory with all fixative combinations. The staining was dark and even, and the heterochromatic segments were easily observed. The slides stained with acetocarmine gave equally good results; however, the slides stained in propionic carmine were of poorer quality. In this latter instance there was more graininess or fuzziness in the chromosomes, and the stain was rather faint. With the Feulgen and the acetocarmine stains the fixatives used did not alter the basic qualities of the preparation. This was largely the case with the propionic carmine stain, except with those materials fixed with LaCour's 2BE fixative. In this instance, the chromosomes were very darkly stained and the H-segments more difficult to distinguish than those chromosomes prepared with any of the Carnoy's fixatives.

Discussion

The results of this study show that in all instances no significant length variation in the chromosome itself or in any of the individual heterochromatic segments is indicated as resulting from the stains or fixatives or stain-fixative combinations utilized. Thus, the matter of procedure to be followed in studies of differential staining patterns of *Trillium* is largely one of individual preference. These data further suggest that the differential staining patterns obtained using one fixation and staining procedure can be easily compared with karyotypes obtained using different stain and fixation procedures.

The quality of the preparations obtained by these various procedures is also noteworthy from the viewpoint of time requirements of the various techniques. The usual procedures followed in work of this type are frequently rather time consuming. Fixation with Carnoy's fixatives usually requires 12-24 hours, whereas fixation with LaCour's 2BE is much quicker, requiring only 15 minutes. The staining procedures are also quite different in time requirements. Staining with acetocarmine or propionic carmine requires only 5-10 minutes before smearing, but the Feulgen reaction requires some 3 hours of work and is considerably more complex than either of the other two staining procedures. For ease and speed of handling, the fixation of cold-treated materials in LaCour's 2BE (15 min) followed by staining in acetocarmine (5 min) is here suggested as a good and convenient procedure to follow.

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