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The Effect of Several Fixing Solutions on the Fixation Image and Staining of the Lilac Leaf (*Syringa Vulgaris*)

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ABSTRACT—The purpose of this study was to determine the effect of several killing agents on samples cut from the lilac leaves (*Syringa vulgaris*). The leaves were cut in four different lengths to indicate the different treatments to be given to them. The killing solutions used were CRAF III, FAA II, Allen-Bouin II and a control with no killing agent. A procedure for embedding in paraffin was used to provide one sample from each of the four treatments on each microscopic slide. The slides were stained with Safranine-0 and Fast-Green; Safranine-0 and Anilin Blue; Safranine-0 and Bismarck Brown and Fast-Green; Safranine-0 and Delafield's Hematoxylin; Conant's Triarch Quadruple and Heidenhain's Iron-Alum Hematoxylin. The sections were examined to note the effect of the four treatments on the fixation image and on the staining. From the standpoints of both the fixation image and staining, the findings indicate preference for treatment in the following order CRAF III, Allen-Bouin II, FAA II and no killing agent.

INTRODUCTION

According to Chamberlain (1) the ideal fixing agent for cell contents has not yet been found. To meet optimum standards it must penetrate rapidly; it must preserve structures perfectly; and it must harden so thoroughly that every detail shall remain unchanged during the subsequent process of dehydrating and clearing, embedding, sectioning, and staining.

Sass (7) states that no single substance has been found to meet the requirements of successful preservation. The formulas used for this purpose consist of ingredients in such proportions that there is a balance between the respective shrinking and swelling

actions of the ingredients.

Several fixing solutions are available. Three formulas commonly used as killing agents were selected for this study comparing the effect they cause on the fixation image and on the staining of the lilac leaf embedded in paraffin, and each of these was compared with a control sample without any fixing agent.

The leaf of the lilac or by its botanical name *Syringa vulgaris* is classified as simple, opposite, ovate and entire (5). Haupt (3) describes the lilac leaf as follows:

> "A cross section of a typical mature leaf reveals the following tissues: the epidermis, usually a single external layer of colorless cells with cutinized outer walls and containing numberous stomata; the palisade tissue, generally comprising one or two layers of green cells, vertically elongated, and lying beneath the upper epidermis; the spongy tissue, a loose region of green cells and large intercellular spaces: the veins, vascular bundles that traverse the spongy tissue. The arrangement of the conducting tissues in the veins is collateral the xylem lying above the phloem. Cambial activity, if present, is weak. The mesophyll including the palisade and spongy tissues, is the photosynthetic tissue of the leaf."

Chamberlain (1) comments the lilac leaf as a good example to illustrate the palisade and spongy parenchyma.

MATERIALS AND METHODS

Freshly cut lilac leaves were placed in water to retain their freshness during a few hours of standing before they were cut for this study. Then they were placed on wet paper towels and the leaves were cut in different lengths as a means of identifying the samples given the four different treatments. These sections were placed in a bottle half filled with water and were aspirated to remove air. All the samples were then transferred into a large shallow container to be sorted out. They were finally placed into four bottles, each bottle containing samples all of one size. The bottles were treated as follows:

1. Leaves cut 1/8″ long—Control, no killing agent was used. They were put directly into the dehydrating procedure of Ethyl Alcohol-Tertiary Butyl Alcohol Series until 50 per cent alcohol was reached.

2. Leaves cut 2/8″ long—These were placed into CRAF III solution. The solu-

tion was changed three times at intervals of one hour. After 24 hours they were placed in TBA Series until 50 per cent alcohol was reached.

3. Leaves cut 3/8″ long—They were placed into Allen-Bouin solution. The procedure was same as those put into CRAF III solution.

4. Leaves cut 4/8″ long—These leaves were placed into water and the water was changed three times. The leaves were placed in FAA II solution.

When the above first three samples were ready to be placed into TBA series of TBA Grade 1, all four samples were combined and put into this solution together. From this point on, the recommended schedule was followed for Ethyl Alcohol-Tertiary Butyl Alcohol Series.

During embedding, one sample from each of the above four procedures was brought together so they could be cut in one block of paraffin and stained together on one slide. Each sample was made to stand with the cut side up, so as to provide transverse sections of each one. They were cut 12 microns thick on the rotary microtome, mounted on Haupt's adhesive, and placed on a warming plate until the paraffin was stretched. They were then allowed to dry thoroughly.

The following stains were used, employing the recommended staining schedule in each case :

1. Safranine-0 and Fast-Green
2. Safranine-0 and Anilin Blue
3. Safranine-0 and Delafield's Hematoxylin
4. Safranine-0, Bismarck Brown and Fast-Green
5. Conant's Triarch Quadruple
6. Heidenhain's Hematoxylin.

RESULTS

Observations concerning the effects of the killing agents on the staining and on the fixation images are presented in the following Tables 1 and 2 respectively.

TABLE 1 Differences in Staining Effects on Sections of the Lilac Leaf (*Syringa Vulgaris*) Embedded in Paraffin, According to Killing Agents Used.

Stain	Tissue	Killing Agents			
		No Killing	FAA II	Allen-Bouin	CRAF III
Saf-O & Fast Green	Mesophyll	Blue-Green	Blue-Green	Green	Green
	Nucleus	Green	Green	Orange-Yel	Red-Orange
Saf-0 & Anilin Blue	Mesophyll	Blue	Blue	Pale Blue	Pale Blue
	Nucleus	Dark Blue	Dark Blue	Orange-Yel	Light Red
Saf-0 & Delafield's Hema.	Mesophyll	Dark Red Purple	Red Purple	Light Purple	Light Purple
	Nucleus	Dark Purple	Dark Purple	Light Red Purple	Light Red Purple
Conant's Tri-Quad.	Mesophyll	Green	Green	Light Green	Light Green
	Nucleus	Yel-Green	Yel-Green	Red-Orange	Dark Red-Orange
Saf-O & Bismarck Brown & Fast-G	Mesophyll	Blue Green	Blue Green	Pale Green	Pale Green
	Epidermal	Light Yel-Green	Yel-Green	Yel-Brown	Yel-Brown
	Nucleus	Dark Green	Dark Green	Yel-Orange	Yel-Orange
Heidenhain's Hema.	Mesophyll	Light Yel-Brown	Light Yel-Brown	Dark Yel-Brown	Dark Yel-Brown
	Nucleus	Black	Black	Greyish-Black	Greyish-Black

TABLE 2 Differences in Fixation Image on Sections of the Lilac Leaf (*Syringa Vulgaris*) Embedded in Paraffin, According to Killing Agents Used.

Stain	Tissue	Killing Agents			
		No Killing	FAA II	Allen-Bouin II	CRAF III
Saf-O & Anilin Blue	Palisade Cells	Shrinkage	Shrinkage	Quite uniform but seemed swollen	Quite Uniform
	Epidermal Cells	Peeling of the lower epidermal seen in several places			
Saf-O & Fast Green	Palisade Cells	Shrinkage	Shrinkage	Swelling	Quite Uniform
Saf-O & Del. Hema.	Palisade Cells	Shrinkage	Shrinkage	Quite Uniform	Quite Uniform
	Epidermal Cells		Peeling of upper cells		
Conant's Tri. Quad.	Palisade Cells	Shrinkage	Shrinkage	Swelling	Quite Uniform
	Epidermal Cells	Peeling			
Saf-O & Bismarck Br. Fast Green	Palisade Cells	Shrinkage	Shrinkage	Swelling	Quite Uniform
	Epidermal Cells		Peeling of upper cells		
Heidenhain's Hematoxylin	Palisade Cells	Shrinkage	Shrinkage	Swelling	Quite Uniform
	Epidermal Cells	Peeling	Peeling		

NOTE: Plasmolysis was noticed in all the samples but was more extreme in the samples treated with no killing agent and those treated with FAA II. Larger intercellular spaces could be seen in the control samples than in others.

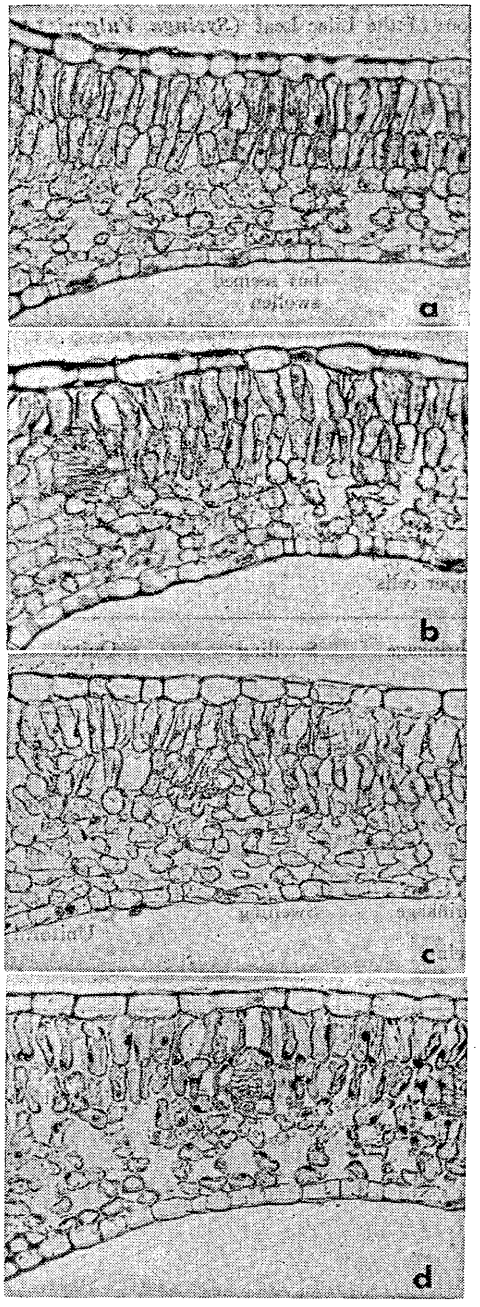

Fig. 1. Lilac leaf treated with different killing agents; a. CRAF III, b. Allen-Bouin II, c. FAA II, d. no killing agent. X 240.

DISCUSSION

The Effect of the Killing Agent on the Fixation Image. Details of these results are given in Table 2 and illustrated in Fig. 1. The observation indicates how important it is that a good fixing solution be used to preserve the tissue in their normal condition. For instance, the sample with no killing agent showed a much poor image when compared to uniform cells of the sample killed with CRAF III solution, the former having extreme plasmolysis and shrinkage of palisade cells and peeling of the epidermal cells. The sample killed in Allen-Bouin solution on the contrary, looked swollen, and it seems likely that perhaps the enlargement of the palisade cells in this solution was due to the picric acid. This chemical enlarges the vacuoles, according to the statements made by Chamberlain (1) and Gray (2).

Sample killed in FAA II showed shrinkage of the palisade cells, plasmolysis and some peeling of the epidermal cells though not as extreme as in those treated with no killing agent. The formula used for FAA II in this study was as follows: water 15 cc., ethyl alcohol 70 cc., glacial acetic 5 cc., formaldehyde 10 cc. Chamberlain (1) suggests that if the protoplasm shrinks

away from the cell walls when 5 cc. of glacial acetic is used with 5 cc. of commercial formalin and 90 cc. of 50% or 70% alcohol, one should increase the proportion of acetic acid to 7% or even to 10%. The small amount of glacial acetic acid in the formula used in this study may have been the cause of the shrinkage of cells.

The Effect of Killing Solution in Staining. Details of these results are given in Table 1. The sample treated with no killing agent and with FAA II seemed to stain intensely and therefore showed poor differentiation when compared to those treated with CRAF III. According to Johannson (4) fixing solutions sometimes interfere with the staining of the specimen causing poor differentiation. An exception to this finding was in the color resulting from Safranine-O which gave a similar red color in the xylem tissue of all samples. When comparing the staining effect on specimen treated with Allen-Bouin II and CRAF III solutions, the color appeared somewhat lighter in shade in the Allen-Bouin solution.

SUMMARY

The effects of three different killing agents on the fixation images and on the staining of the lilac leaf have been observed. The killing agents used were CRAF III, Allen-Bouin II, FAA II, and no killing agent as a control. The best killing agent seemed to be CRAF III, and the samples treated without any killing agent gave the poorest results.

Lee (6) in his book "The Microtomist's Vade-Mecum" in considering botanical technique, lists fixing solutions in the following order of preference : - Flemming and its modifications, Navashin, Chromo-acetic, Bouin, Aceto-formalin, alcohol formalin alcohol. The writer would arrange the agents used in this study in the order following of preference : - CRAF III, Allen-Bouin II, FAA II and no killing agent. It is interesting to note that this order of preference seems to agree largely with the order of preference given by Lee. This preference is from the standpoint of both fixation image and staining effect resulting from the killing agent used.

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