Observational and Experimental Studies of Sensitive Plants.

X. On the Fixation of Threadlike Apparatus in Cortex of Petiole*

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鳥山英雄**: オジギソウの研究 X. 葉柄皮層における紐状装置の固定について

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The author recently reported that in the petiole of *Mimosa pudica* L. there are threadlike apparatus which have a connection with tannin vacuoles (Toriyama 1955). These threadlike apparatus have been hitherto overlooked or ignored by plant anatomists or physiologists, both in the study on living and fixed materials. During the past three years, the author has made an effort to get data which are of immediate relevance to the case of this threadlike apparatus. In the previous paper, the data concerning vital staining of the threadlike apparatus was described (Toriyama 1957). The purpose of the present work was to obtain a more complete knowledge of the nature of the threadlike apparatus by means of fixing methods together with some experiments.

This study has made, using materials similar to those, reported in paper VII (1957 *l. c.*). In order to observe the parenchyma in the petiole before the bending movement, the plants were as usual exposed to ether vapour for 20 minutes, thus rendering the petiole of these plants incapable of responding to any stimuli. The material in the present investigation consisted chiefly of those plants which had not received any stimuli. The fixing methods were made on the petiole to examine their cytological reactions. The details shall be described in the following paragraphs.

Observations and Experiments

a) *Results through classical cytological methods.* Results obtained by classical cytological methods are briefly summarized in the remarks of table 1. The detailed explanations concerning these results are described as follows under the topics of fixatives employed.

(1) *Kaiser's fluid.**** After having fixed for 5 hours in this fluid, the material was directly removed to 70 per cent alcohol, added a few drops of iodine solution, and then to absolute alcohol, butyl alcohol, butyl alcohol paraffin successively, and finally embedded in paraffin as usual. Paraffin blocks were cut 10 micra in thickness. The

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*** Kaiser's fluid is composed of 10 g. of sublimate, glacial acetic acid 3 ml. and distilled water 300 ml.,

Table 1

Fixatives	Result of fixation	Remarks
Kaiser's fluid	+	slender
Susa's fluid	+	slender rosary-like
Bouin's fluid	+ +	slender rosary-like
Neutral formalin	+	slender
Müller's fluid	+	rosary-like
Carnoy's fluid	—	
Bensley's fluid	+ +	slender
Champy's fluid	+ +	slender
Altmann's fluid	+ +	slender

sections were stained with 0.5 per cent alcoholic toluidine blue solution which was very convenient for differentiating the threadlike apparatus and the tannin vacuole. Kaiser-toluidine blue preparation showed a good result (Fig. 1). The thread and tannin vacuoles stain greenish blue by this dye solution, assuming a similar status to the apparatus moderately stained with vital staining (Toriyama 1957 *l. c.*). The cell wall, cytoplasm, nucleus, chloroplast and other cellular elements are not however stained with this solution.

(2) *Susa's fluid*.* The proper duration of the fixation was about 24 hours. Fixed material was then washed out with 95 per cent alcohol. The dehydration was performed through a series of tertiary butyl alcohol, and was finally embedded in paraffin as usual. Paraffin blocks were cut 10 micra in thickness. The sections were stained with 0.5 per cent alcoholic toluidine blue solution. The threadlike apparatus in a rosary-like form stains very intensely blue. The toluidine blue staining revealed good results concerning the thread and tannin vacuole. But the only regrettable fact was that the cell shrink a little (Figs. 2a, b and 3a, b).

(3) *Bouin's fluid*** The moderate duration of fixation was about 4 hours usually. After fixation the material was washed in running water overnight. The dehydration and cutting procedure were performed in the same manner as in the foregoing method. Then, the sections were stained with alcoholic toluidine blue solution. By this technique, the threadlike structure appeared very clearly (Figs. 4, 5, 6). Sometimes, the threadlike apparatus appeared a little broader than those in the other preparation (Figs. 5, 6).

* Susa's fluid is composed of 50 g. of mercuric chloride, 2 g. of trichloroacetic acid, 20 ml. of formalin, 4 ml. of glacial acetic acid and 30 ml. distilled water.

** Bouin's fluid is composed of 75 ml. of picric acid, 25 ml. of formalin and 5 ml. of glacial acetic acid.

Figs. 1-6. Threadlike apparatus in the lacunal parenchymatous cell of petiole. $\times 800$. 1-fixed with Kaiser's fluid; 2a, b and 3a, b-fixed with Susa's fluid; 4($\times 320$) 5, 6-fixed with Bouin's fluid; All materials are stained 0.5% alcoholic toluidine blue. ta-tannin vacuole; th-threadlike apparatus; n-nucleus.

Figs. 7-12. Threadlike apparatus in the parenchymatous cell of petiole. $\times 800$. 7-fixed with 10% neutral formalin; 8-fixed with Müller's fluid. Both materials are stained 0.5% alcoholic toluidine blue. 9, 10-fixed with Bensley's fluid; 11a, b-fixed with Champy's fluid; 12a, b-fixed with Altmann's fluid. Arrows indicated the threadlike apparatus. n-nucleus; th-threadlike apparatus; ta-tannin vacuole; ch.-chloroplast.

(4) *Neutral formalin.* The commercial formalin neutralized with calcium carbonate was diluted with distilled water in proportion of 1:5. The proper duration of fixation by this fixative was 24 hours. The washing and dehydration procedure are performed in the same manner as in the foregoing methods. The material was then cut 10 to 15 micra in thickness. The sections were stained 0.5 per cent toluidine blue solution. The adequate duration of fixation in the dye solution was determined only from experience. For the present material 10 hours staining in the dye solution was quite enough to stain the threadlike structure and the tannin vacuole. It must be noted that the thread assumes a slender feature by the fixation of neutral formalin (Fig. 7).

(5) *Müller's fluid.** The adequate duration of fixation was about 10 hours. After fixation the material was washed in running water overnight. The dehydration and cutting procedure were performed in the same manner as in the foregoing methods. The sections were stained with alcoholic toluidine blue as usual. The thread and tannin vacuole were also found to be well preserved by Müller's fluid. The thread and the tannin vacuole were equally stained in a greenish blue color. It is remarkable that the thread takes a rosary-like feature as shown in Fig. 8.

(6) *Carnoy's fluid.*** The material was fixed in this fluid for 5 hours. After completion of the fixation the material was dehydrated in absolute alcohol. The cutting and staining procedure were performed as usual. By this technique, the cell wall and the cytoplasm stained light blue. After this fixing procedure the threadlike apparatus and tannin vacuole were found to be mingled with each other, both being stained light blue color. Consequently, the fixing figure of the thread and tannin vacuole were not observable.

The available data from the experiments concerning the fixing of threadlike apparatus may be summarized as follows. Among various fixatives, such as Kaiser's fluid, Susa's fluid, Bouin's fluid, Müller's fluid and simple neutral formalin used, the best results were obtained by using Bouin's fluid.

b) *Results through new methods.* Other than the above mentioned methods, the author attempted the following methods using osmium tetroxide. With a purpose to know the fine structure of the thread, the author tried new methods upon the petiole. First he employed Bensley's fluid.*** Small pieces of tissue were kept in the fixing fluid, the adequate duration of fixation being about 10 hours. After completion of fixation they were thoroughly washed in running water for 10 hours to remove any traces of the reagents. These materials were cut 20 to 40 micra in thickness by means of a hand microtome. The sections were washed in distilled water, which was

* Müller's fluid is composed of 2.5 g. of potassium bichromate, 1 g. of sodium sulphate and distilled water 100 ml.

** Carnoy's fluid is composed of 60 ml. of absolute alcohol, 30 ml. of chloroform and 10 ml. of glacial acetic acid.

*** Bensley's fluid is composed of 4 ml. of 2.5 % aqueous sublimate, 1 ml. of 2 % osmium.

changed for several times. The sections were mounted in pure glycerin, without any subsequent staining. This technique gives not only excellent result in demonstrating the thread, but also is outstanding in the fixation of nuclei. By dint of this technique, the threads were clearly observable even before reception of stimuli in the parenchymatous cell. The threads in this case have a tendency to form a cluster around the nuclei as shown in figures 9 and 10. After receiving a stimulus, the same figure was still observable in the cell. In addition, these threadlike apparatus were also found in the parenchyma of the transitional portion from the petiole to primary pulvinus. Figure 10 shows the apparatus of this portion, the material being fixed with Bensley's fluid. As the thread of these materials was very favorable for detailed study, the author used this technique for the observation of various features of the threads.

Next, the material was fixed with Champy's fluid.* The adequate fixation with this fixative was only known from experience. For the present materials, 20 hours-fixing in Champy's fluid was quite enough to fix the thread in the cell. After fixation the material was thoroughly washed in running water for 10 hours to remove any trace of the fixing reagents. These materials were then cut 20 to 40 micra in thickness. The sections were also mounted in pure glycerin. By this fixing fluid, the thread does not assume the rosary-like form (Figs. 11a, b). The thread and tannin vacuole are tinged in black color by osmic acid, while the nuclei are colored light brown. The thread assumes various features. Sometimes they develop to the extent of assuming a long slender threadlike apparatus. Before receiving the stimulus, the cytoplasm and the threadlike apparatus were not mixed with each other, as in the case of Carnoy's fluid. After being stimulated the same figure was still observable in the cell.

Thirdly, the material was fixed with Altmann's fluid.** The fixing and washing procedure were performed in the same manner as before. By this technique, the difference in the parenchymatous cell before and after the bending movement could not be observed. The threads appear uniformly in the form of slender threads. As an effect of the fixation, the threads have a tendency of gathering themselves in a cluster around the nuclei as shown in Figures 12a, b. These threads are blackened by the osmium tetroxide. The cytoplasm is not markedly differentiated from the cell wall. Both the threads and the tannin vacuoles were stained black within 10 hours, showing no differences color. The available data from the above mentioned results may be summarized as follows. The threadlike apparatus stains black by osmium tetroxide, and takes a slender form.

The available data on the fixation concerning the threadlike apparatus in the lacunal parenchyma must be mentioned as follows. Contrary to my expectation, the

* Champy's fluid is composed of 35 ml. of 3 % aqueous solution of potassium bichromate, 35 ml. of 1 % chromic acid solution and 20 ml. of 2 % osmium.

** Altmann's fluid is composed of 10 ml. of 5 % aqueous potassium bichromate and 10 ml. of aqueous 2 % osmic acid.

threads do not join one cell with another cell lengthwise, nor do they connect with the lateral neighbouring cells.

Discussion

Many attempts have been made in order to make clear the morphology of the canaliculi or vacuome in the plant cells. Among these attempts the successful observations of the canaliculi have been obtained upon the meristem cells (Holmgren 1899, Guilliermond 1922, Gicklhorn 1926 *et. al*). During course of the study reported in the fourth paper of the present series of investigations, the author found a threadlike structure in the lacunal parenchyma of *Mimosa* petiole. Recently, the author has reported that basic dyes stain the thread in the cortex of the petiole before and after receiving the stimulus. It was confirmed that the threads possess a lipoidal nature from the staining properties with vital dyes. Furthermore, the author postulates that the thread is one of cellular elements, and is not mere artificial figure (Toriyama, 1957 *l. c*).

In order to know the further detailed cytological and chemical natures of the apparatus, the author attempted to use some fixing techniques. It seems important for this purpose that the fixatives adopted should list the form of the apparatus as modified artificially or solved by the reagents. Various fixatives, such as Kaiser's fluid, Susa's fluid, Bouin's fluid, Müller's fluid, Carnoy's fluid, or simply 10 per cent neutral formalin were used. Among them the best results were obtained with the combination of Bouin's-toluidine blue. By using alcoholic toluidine blue, the tannin vacuole and threadlike apparatus were stained very strongly and the contour of the thread had a clear-cut appearance. By means of the author's new technique, the analysis of their finer architecture became easy. In other words, the fixatives containing bichromate and osmic acid have proved to be excellent for this purpose. Osmic acid on the other hand stains thread positively. This reaction probably shows that the accumulation of tannin substance occurs in the thread. It would not be too much to say that the functional significance of a threadlike apparatus is the accumulation of the tannin substance. Therefore, these two morphologically different kinds of elements should be placed in the same category. In other words, the threadlike apparatus may be called a threadlike vacuome, which reminds us of the filamentous vacuole as reported by Bailey (1930). Zirkle (1932) also observed the canal and net-like vacuole in *Pinus Strobis*. These vacuoles and that of the *Mimosa* may be placed in different categories.

The author found that the threads assume rosary-shapes or slender threads in the living condition as shown by the vital staining. These threads are, on the other hand, capable of bending both under the natural as well as under the artificial condition (Toriyama 1957 *l. c.*). In the fixed material, their shapes show many modifications, and often shows to be curious. In some cells *e. g.* those of the parenchyma of the transitional tissue, the threadlike apparatus usually appear attached to the

nucleus. Sometimes chloroplasts draw near to the threadlike apparatus, showing a close relationship between the two (Figs. 11a, b). These phenomena seem to be very suggestive.

The main obstacle to the chemical investigation has been so far the difficulty of obtaining a pure sample of the threadlike apparatus and tannin vacuole. The detailed chemical nature and the distribution of these threads are expected to be the subjects of future research. Furthermore, the questions of the structure and function of the threadlike apparatus remain unsolved for future studies.

Summary

In the present investigation, the modes of the fixation of the threadlike apparatus in the cell of *Mimosa* petiole were studied from the cytological view-point. The experimental results obtained are summarized below.

1) Threadlike apparatus and tannin vacuole in the lacunal parenchymatous cell of the petiole are chiefly dealt with.

2) Threadlike apparatus and tannin vacuole were fixed with Kaiser's fluid, Susa's fluid, Bouin's fluid, Müller's fluid and neutral formalin. They were stained with a 0.5 per cent alcoholic solution of toluidine blue. The combination of Bouin's fluid and toluidine blue was found to be a favorable and a specific method for staining the thread and tannin vacuole.

3) By Carnoy's fluid, the thread and tannin vacuole are inadequately fixed, and are dissolved and mixed with each other.

4) By fixing the lacunal parenchyma of the petiole with the fixatives containing osmium tetroxide, *i. e.* with Bensley's, Champy's and Altmann's fluid, the thread and tannin vacuole are observed in black staining.

5) Judging from the data obtained by these experiments, it is concluded that the thread is one of the cellular elements, and is by no means a mere artificial figure.

6) The threadlike apparatus do not join one cell with another cell lengthwise, nor connect the lateral neighbouring cells.

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摘 要

オジギソウ (*Mimosa pudica* L.) の葉柄の皮層、とくに細胞間隙が大きく発達している部分の柔細胞中にはタンニン液胞と紐状装置が認められるが、これらは塩基性色素で生体染色することができる (Toriyama 1955, 1957)。この紐状装置は次に述べる方法によっても明瞭に観察することができる。Kaiser 液, Susa 液, Bouin 液, Müller 液, およびホルマリンで固定した後パラフィン切片とし、これらをそれぞれトリジンブルーのアルコール溶液 (0, 5%) で染色する。この方法によると細胞膜や細胞質は染色されず、紐状装置とタンニン液胞が青色に染色される。Carnoy 液ではこれら紐状装置とタンニン液胞は固定されない。また筆者創案の方法、すなわち、Bensley 液, Champy 液, Altmann 液等で固定した材料を水洗した後、ハンドミクロトーム等で切片とし、染色を行わずにただちにグリセリンに封じて検鏡する。この方法は細胞膜および細胞質は染色されていないので、細胞内の観察に好都合である。紐状装置およびタンニン液胞はオスミウムによって黒色に固定され、非常に明瞭に認めることができる。以上の観察によると紐状装置は人工産物ではなく一つの細胞要素と考えられる。

Short Communication

Über den lichtunempfindlichen Teilprozess in der blühinduzierenden Dunkelperiode bei *Pharbitis Nil* (Vorläufige Mitteilung)

von Atsushi TAKIMOTO,* Katsuhiko IKEDA* und Shun-ichiro IMAMURA*

滝本 敦* 池田勝彦* 今村駿一郎*: アサガオの花成の促進される暗期における
比較的光に安定な過程について (予報)

Eingegangen am 15, August 1958

Es ist bekannt, dass bei vielen Kurztagpflanzen die auf schwaches Licht von genügender Dauer folgende Dunkelperiode auf die Blütenbildung unwirksam ist.^{1), 3)} Bei *Pharbitis Nil* trifft dies ebenfalls zu. Wenn aber die Dauer der Vorbeleuchtung mit schwachem Licht abgekürzt ist, nämlich bis auf 4 Stunden, kommen die Pflanzen, selbst nach einer Dunkelperiode von subkritischer Dauer, zur Ausbildung der Blütenanlagen. Der Versuch wurde mit Keimlingen von *Pharbitis Nil*, Sorte, „Violett“, ausgeführt, die durch eine einzige induktive Dunkelperiode zur Blütenbildung gebracht werden können²⁾. Die kritische Dunkelperiode unter den günstigsten Bedingungen ist etwa 9 Stunden.

Die Samen wurden mit konzentrierter Schwefelsäure 20 Minuten lang behandelt, gründlich gewaschen und darauf im Leitungswasser zur Quellung gebracht. Nach 2 Tagen wurden die gleichmässig keimenden Samen in Töpfen ausgesät, und im Gewächshaus bei $30 \pm 2^\circ$, unter Dauerlicht (natürliches Tageslicht und nächtliche Beleuchtung

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