Male Sterility Caused by Cooling Treatment at the Young Microspore Stage in Rice Plants

XX. Optical microscopical observations of unfixed, intact anthers

Iwao Nishiyama
(Hokkaido National Agricultural Experiment Station, Hitujigakke, Sapporo 061-01)
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It has been generally considered in studies on the tapetum and microspores that fixation is a prerequisite to microscopical observations, and a number of methods have been advanced to fix anthers with minimum artifacts. However even the best methods of fixation can not be free from artifacts.

Microscopical observations of anthers in their intact states have an advantage of being exempt from artifacts, though they can not be applied to detail cytological structures. This paper shows that observations of intact anthers are useful in the estimation of microspore size, as well as in the studies of microspore and tapetal abnormalities.

Materials and Methods

Twenty seeds of the variety “Hayayuki” were sown in a circle in each of 1/5,000 a pot. Rice plants were grown in natural light rooms of the phytotron at 24°C day-19°C night throughout the entire growth period except during cooling treatment. The cooling treatment was made at 12°C for 4 days at the young microspore stage (the critical stage to cool temperature damage). The fertility of the control plants was more than 90 percent, and that of the cooled was ranged approximately 10 to 30 percent.

For the observations of tapetal hypertrophy, and the studies on the relation between microspore stage and its diameter, anthers were excised from the flowers at the proper stages which were judged by means of the auricle distance method and flower length. In the case of cooled plants, the excision was made at the end of the 4-day cooling treatment.

For the estimation of distribution of microspore diameter, anthers were excised from flowers of 4.0–4.5 mm in length. Anthers of cooled plants were taken one day after the end of the cooling.

For glutaraldehyde fixation, the tips of spikelets and anthers were excised, and the spikelets were plunged into an ice cold 5 percent glutaraldehyde solution (buffered to pH 7.0 by 0.1 M phosphate solution), aspirated for about 30 minutes, and then kept for 24 hours at 4°C. After the fixation, the spikelets were dehydrated through a tertiary butyl alcohol series, embedded in paraffin, sectioned and stained with methyl green-pyronin Y solution.

For intact observations, excised anthers were mounted on a slide glass with 0.7 percent sodium chloride or 0.25 M sucrose solution, and observed under optical microscope. The developmental stages of microspores were estimated according to Satake’s classification, and the microspore phases were further subdivided by the degree of pore formation, exine thickening and vacuole size, that is, early early (EE), middle early (ME), late early (LE), and so forth.

Results

Fig. 1 shows a comparison of fixed and unfixed normal microspores at the tetrad stage. Microspores were shrunk and the cytoplasm was coagulated by the fixation with a glutaraldehyde (GA) solution (Fig. 1–1). On the other hand, moving cellular
particles were clearly observed in the intact microspores (Fig. 1–2).

Fig. 2 compares fixed and unfixed normal microspores at the late early phase. Shrinkage occurred in the course of GA fixation, though pores and nucleoli are more clearly seen with staining (Fig. 2–1). In the intact microspores, pores are just discernible (Fig. 2–2).

The degree of shrinkage with GA fixation ranged from approximately 10 to 30 percent in the microspores at the tetrad or the late early phase. Further higher degree of shrinkage was observed in the case of fixation with Carnoı̈ solution.

Figs. 3–6 show abnormal microspores and Figs. 7–13 tapetal cells or layers, in unfixed intact materials.

Figs. 3 and 4 are disfigured microspores resulted from the abnormal divisions of meiosis. Big, small, ellipsoidal, and incompletely separated microspores are seen. Figs. 5 and 6 show other microspore abnormalities. The microspore shown in Fig. 5 has an angle. The cause of the angle is not known. The microspores in Fig. 6 have ruptured exine. The cell membranes of those cells are still intact and clearly visible under microscope.

Fig. 7 shows a part of a normal tapetal layer at the tetrad phase of microspore. Fig. 8 is a typical medium-sized hill-type dilatation of tapetum, and Fig. 9 is a part of a wave-type dilatation. The wave-type is newly named in this paper. This is a variety of the hill type.

Fig. 10 shows a wall-type dilatation, which separates the anther locule into two parts. This type is newly named in this paper, and is considered a variety of the balloon type.

Figs. 11 and 12 are big and small balloon-type dilatations. Big balloon-type dilatations have, in many cases, very sparse inclusions, and thus they are barely discernible with thin boundary membranes (cell mem-

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Middle early phase</th>
<th>Early early phase</th>
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<tbody>
<tr>
<td></td>
<td>Larger Locule**</td>
<td>Smaller locule**</td>
</tr>
<tr>
<td>15.0 μm*</td>
<td>0.0%</td>
<td>10.0%</td>
</tr>
<tr>
<td>16.5</td>
<td>0.0</td>
<td>15.0</td>
</tr>
<tr>
<td>18.0</td>
<td>28.3</td>
<td>43.3</td>
</tr>
<tr>
<td>19.5</td>
<td>63.3</td>
<td>25.0</td>
</tr>
<tr>
<td>21.0</td>
<td>8.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Average</td>
<td>19.2 μm</td>
<td>18.1 μm</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.9</td>
<td>1.6</td>
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* 15 μm means a range of 15 ± 0.75 μm (14.25–15.75 μm).
** A larger and a smaller locules of an anther.
*** Two larger locules of an anther.
Fig. 7~13. Unfixed tapetal cells or layers.

Fig. 7. Normal tapetal layer (*) (tetrad). ×400. Fig. 8. A hill-type dilatation (arrow) (tetrad). ×400. Fig. 9. A part of wave-type dilatation (late early phase). ×800. Fig. 10. A wall-type dilatation (arrow) (middle middle phase). ×400. Fig. 11. A big balloon-type dilatation (arrow) (tetrad). ×400. Fig. 12. A big (arrow) and a small (0) balloon-type dilatations (late early phase). ×800. Fig. 13. A cluster of tapetal material originated from balloon-type dilatation (0) (late early phase). ×800.
branes) (the arrow in Fig. 12). Fig. 13 shows a cluster of tapetal materials originated from a balloon-type dilatation.

The distribution of microspore diameter in the locule was estimated for unfixed intact materials and is shown in Table 1, for two normal anthers. Larger locules have slightly larger microspore diameters than smaller locules do. This due to the fact that the developmental stage is slightly more advanced in larger locules than in smaller locules. The distribution is extremely uniform, particularly in the larger locules. Table 2 shows the distribution of microspore diameter in two anthers from the plants cooled at the critical stage to sterility (the young microspore stage\textsuperscript{3}). The distribution ranges widely from 10.5 μm to 30 μm and the standard deviation was more than 3 times of that in the normal anthers (refer to the data in Table 1). The average diameter is slightly larger than that in the normal anthers. Observations showed that these irregularities are primarily due to uneven or failed dividing in the meiotic division.

Fig. 14 shows changes in microspore diameter with developmental phases. After the meiosis of pollen mother cells, the diameter sharply decreases to approximately 16 μm at the tetrad phase. Then it gradually increased and was approximately 32 μm at the beginning of the late microspore phase. The diameter of microspores in cooled anthers showed no clear difference from that of normal ones.

**Discussion**

The primary objective of this paper is to show that the optical microscopical observation of the anther in its intact state is fruitful in some particular studies. The usefulness of intact observation has been neglected a long time since the development of excellent staining methods.

Observations in intact states are exempt from all artifacts which are produced in the course of fixation, dehydration and embedding. This is an extremely valuable point for some specific purposes, such as the estimation of microspore volume, though intact observation can not reach detailed structures which are uncovered by staining methods.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Anther I</th>
<th>Anther II</th>
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<tbody>
<tr>
<td>10.5 μm*</td>
<td>1.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>12.0</td>
<td>0.0%</td>
<td>0.0</td>
</tr>
<tr>
<td>13.5</td>
<td>0.0%</td>
<td>3.3</td>
</tr>
<tr>
<td>15.0</td>
<td>8.8%</td>
<td>12.5</td>
</tr>
<tr>
<td>16.5</td>
<td>15.0</td>
<td>6.7</td>
</tr>
<tr>
<td>18.0</td>
<td>15.0</td>
<td>25.0</td>
</tr>
<tr>
<td>19.5</td>
<td>15.0</td>
<td>30.0</td>
</tr>
<tr>
<td>21.0</td>
<td>18.0</td>
<td>5.0</td>
</tr>
<tr>
<td>22.5</td>
<td>8.8%</td>
<td>2.5%</td>
</tr>
<tr>
<td>24.0</td>
<td>11.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>25.5</td>
<td>1.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>27.0</td>
<td>3.8%</td>
<td>0.0%</td>
</tr>
<tr>
<td>28.5</td>
<td>1.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>30.0</td>
<td>1.3%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Average</td>
<td>20.1 μm</td>
<td>18.2 μm</td>
</tr>
<tr>
<td>S.D.</td>
<td>3.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\* 10.5 μm means a range of 10.5±0.75 μm (9.75—11.25 μm).
Fig. 14. Relation between microspore stage and its diameter.

○: Control
×: Cooled at 12°C for 4 days
M1: The first division of meiosis
MII: The second division of meiosis
T: Tetrad
EE: Early early phase
ME: Middle early phase
LE: Late early phase
EM: Early middle phase
MM: Middle middle phase
LM: Late middle phase
EL: Early late phase
ML: Middle late phase
E: Early phase
M: Middle phase
L: Late phase

Another merit of intact observation is that there is no need of the labor-consuming procedures from fixation to mounting. The observation can be made instantaneously at the time and on the spot of the problem. From this viewpoint, a quick method to estimate sterility at a time shortly after the critical stage is expected to be developed.

For our studies, microspores and tapetal layers around the critical young microspore stage, either normal or abnormal, can be vividly observed in their intact state under microscope (Figs. 1-13). The intact observation of anthers, however, has the following short points: 1. Only two locules which are located upside facing to the cover glass under microscope can be observed. 2. For tapetal layer, only its parts which are located in parallel with the light path can be observed. 3. Photographed pictures are usually not very distinct, because of no staining.

The image of anther was not distinct under microscope when such fixatives as glutaraldehyde was used, or when autolytic degeneration occurred being induced by, for example, cool temperature treatment at the critical stage.

The estimation of microspore diameter should be a good example to show the merit of intact observation (Table 1 and 2, Fig. 14). Microspores are shrunk by fixatives, and the degree of shrinkage is different among different developmental phases or among different fixatives. YAMADA estimated microspore and pollen diameter in the material fixed with 10 percent neutral formalin solution. The data is much smaller than that of intact microspores reported in this paper, though they are roughly parallel (Varieties used were not the same).

Thus, information obtained from fixed materials should be recognized to be with inevitable artifacts even though it is largely valuable. For example, classifications of pollen developmental stages on the basis of fixed figures are still undoubtedly useful. However, considerations into physiological processes based on the fixed images may be sometimes misleading.

Summary

Fresh anthers around the young microspore stage were observed under optical microscope, for the rice plants either of control or cooled at the young microspore stage. Normal and abnormal microspores, and normal and dilated tapetal layers were shown in their intact state (Figs. 1–13). A comparison of fixed (with a glutaraldehyde solution) and unfixed materials showed the shrinkage and disfigurement by the fixation (Figs. 1 and 2).
The distribution of microspore diameter in the locule was estimated in fresh anthers. The distribution was extremely uniform in the normal anthers, while it showed a wide range in the cooled anthers (Tables 1 and 2). The change of microspore diameter was followed with the growth in fresh anthers (Fig. 14).

The merits and restrictions in fresh observation of anthers through optical microscope were discussed.

References


[和文摘要]

イネの小胞子初期冷温処理による雄性不稔

第20報 固定しない試の光学顕微鏡観察

西山 岩男

（北海道農業試験場）

正常なイネ、および冷温処理したイネから採取した小胞子初期前後の試を、固定せず生のまま光学顕微鏡で観察した。この方法で、正常な小胞子、ダーブネ組織、冷温処理により異常となった小胞子、肥大したダーブネ組織などがはっきりと観察しうることがわかった（第1〜13図）。また、固定（グルタルアルデヒド）した小胞子と未固定の小胞子との比較により、固定による収縮、変形がしつつされた（第1、2図）。

未固定の誘引することにより、小胞子の大きさを正確に測定した。1誘引における小胞子の直径は、正常な誘引では非常に均一であるが、冷温処理をした誘引ではびらびらと変異が認められる（第1、2表）。生育にともなう小胞子の直径の変化を測微分精度から小胞子生後期まで測定した（第14図）。

以上の観察結果から、試を固定しないで観察することの有用性、利点、欠点などについて論議した。