Improved Fixation and Embedding Methods for Electron Microscopy of Alternaria alternata Spores

Pyoyun Park*, Tohgo Ohno*, late Syoyo Nishimura**, Kentaro Tanabe**, Keisuke Kohnoto*** and Hiroshi Otani***

Abstract

Attempts were made to improve fixation and embedding methods for electron microscopy of Alternaria alternata spores. Among 16 methods tested, the ultrastructural images were greatly improved by the use of an en bloc alkaline bismuth stain or an osmium tetroxide-potassium ferrocyanide (reduced osmium) fixative. The cellular membranes were highly contrasted even in unstained sections obtained from en bloc alkaline bismuth-stained spores after prefixation in aldehyde and postfixation in potassium permanganate. The use of the reduced osmium, followed by en bloc uranyl acetate staining, resulted in fine and enhanced staining of cellular membranes after primary fixation of spores in aldehyde. An en bloc alkaline bismuth also selectively reacted with the cell wall and plasma membrane in aldehyde and osmium tetroxide-fixed spores. This indicates that the stain is useful for observation of cell wall and plasma membrane. Spores fixed with the reduced osmium always exhibited a poor infiltration of Spurr resin. This was considerably improved by placing the reduced osmium-fixed spores in a resin/propylene oxide mixture at 60 °C for 3 hr, rinsing in propylene oxide, and performing the infiltration-process with prolonged exposure of the spores to resin.

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Key words: Alternaria alternata spores, en bloc alkaline bismuth staining, osmium tetroxide-potassium ferrocyanide fixation, improved embedding methods.

INTRODUCTION

Spores of Alternaria alternata are indeed troubling fungal organisms in electron microscopy because a conventional double fixation method with glutaraldehyde and osmium tetroxide gives no good results for the fine structure. The authors often had tried to observe the spores with an electron microscope in studies of Japanese pear leaves infected with A. alternata, but often failed to preserve their fine structure. Besides, the spores exhibited poor infiltration when they were embedded with a high viscosity Epon resin because of their thick cell wall with abundant chitin. The insufficient infiltration of the resin causes problems such as poor polymerization and difficult sectioning of the resin blocks. In this work, the authors attempted to improve both fixation and infiltration methods using a low viscosity Spurr resin. Preliminary reports describing a part of the improved fixation methods were presented.

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MATERIALS AND METHODS

The Japanese pear pathotype of *A. alternata* was grown in Richards’ medium at 20–25°C for 4 weeks. The mycelial mats were harvested after 4 weeks and rinsed with tap water for 6–8 hr. The mats were then placed on filter paper in a moist chamber at 20°C for 2 days in the dark. Under these conditions, abundant spores were collected[14].

**Fixation.** Sixteen methods for preparing spores in electron microscopy were tested. The procedures are outlined in Table 1. The following fixatives were used: Karnovsky’s fixative (GA)[6]; 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 (OS); 1% tannic acid in 0.1 M cacodylate buffer at pH 7.2 (TA)[15]; 1% osmium tetroxide aqueous solution containing 1.5% zinc iodide (ZIO)[4,5]; 2% osmium tetroxide in 0.1 M imidazole buffer at pH 7.5 (OSI)[2]; Karnovsky’s fixative containing 0.7% ruthenium hexamine trichloride (GAH)[6]; 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 containing 0.7% ruthenium hexamine trichloride (OSH)[6]; 0.5% potassium permanganate aqueous solution (KM)[1,10]; 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.8% potassium ferrocyanide (OSF)[9]; Karnovsky’s fixative containing 0.1% tannic acid (GT1)[17]; Karnovsky’s fixative containing 2% tannic acid (GT2)[17]. All the steps of fixation were performed at room temperature for 0.5–24 hr. The fixatives used were buffered with 0.1M cacodylate buffer at pH 7.2 except for ZIO, KM and OSI. After fixation of the spores with each of fixatives, the specimens were rinsed in 3 changes of the same buffer or water of 10 min each. Some specimens were exposed to 0.1% chitinase (0.1 M MES, pH 6.0) for 30 min at room temperature after briefly prefixing the spores with GA in order to make the post fixative (OSF) easily penetrate into the fungal cells (method o).

**En bloc staining.** Two en bloc stains, 2% uranyl acetate aqueous solution (U)[18] and alkaline bismuth solution diluted 20–40 times from the stock solution (AB)[14] were used. Specimens in methods b and m–p were en bloc stained with U for 2 hr at room temperature after fixation and in methods g and j–l they were exposed in AB for 2 hr at 40°C after fixation.

**Dehydration.** The specimens in methods a–l were briefly dehydrated with a graded series of ethanol (ET) in the time schedule of a routine manner (Table 1). On the other hand, the specimens in methods m–p were dehydrated by prolonging the time of exposure with ethanol.

**Infiltration and polymerization for Spurr resin embedding.** Spurr resin[16] which penetrates deeper into *A. alternata* spores than Epon resin was always used as an embedding medium. After dehydration, the specimens of methods k and n–p were placed in a mixture of 20% propylene oxide and 80% Spurr resin containing accelerator S-1 (PR1) and incubated in a polymerizer at 60°C for 3 hr[17]. The spores were then rinsed with propylene oxide for 30 min at room temperature to remove the semi-polymerizing resin which had been formed in the spores. This step is required to exclude the deteriorated resin which originated due to the strong oxidizing properties of the osmium-ferrocyanide fixative (OSF)[17]. The specimens of methods a–j and m were infiltrated in Spurr resin by a conventional routine method: they were exposed to a mixture of 50% propylene oxide and 50% Spurr resin containing S-1 (PR2) for 6 hr at room temperature and then placed in pure Spurr resin containing S-1 (RE2) for 24 hr at 4°C. The specimens of methods k, l, and n–p were also carefully infiltrated in Spurr resin. They were exposed in a mixture of 50% propylene oxide and 50% Spurr resin without S-1 (PR3) for 6 hr at room temperature. In difference to the conventional method, the infiltration of the spores with pure Spurr resin without S-1 (RE1) was performed for 48 hr at 4°C with an exchange of resin after 24 hr. The specimens were then exposed in pure Spurr resin containing S-1 (RE2) for 48 hr at 4°C with a change of resin after 24 hr. All specimens were polymerized at 60°C for 48 hr.

**Observation.** Ultrathin sections were cut on a Porter Blum MT-1 using a diamond knife and mounted on Formvar-coated or uncoated copper grids. Sections usually were viewed after staining of 2% aqueous uranyl acetate (Uranium) for 20 min and Reynold lead citrate...
Table 1. Comparative fixation, dehydration and resin-infiltration

<table>
<thead>
<tr>
<th>Method</th>
<th>Fixation</th>
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<th>Dehydration with</th>
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<tr>
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<td>3rd</td>
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<td>b</td>
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<tr>
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<td>d</td>
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<tr>
<td>p</td>
<td>GT1 (4 hr) OSF (4 hr) OSF (4 hr)</td>
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</table>

a) All steps of preparing the fungal specimens were carried out at room temperature except for the
b) The explanation of abbreviation used in this table is described in the text of Materials and Methods.
c) The figures in brackets represent the time and temperature in the steps of preparing the specimens.

(Lead) for 10 min at room temperature in JEOL 100-S or 1200-EX electron microscopes. Some
sections were stained with alkaline bismuth solution (Alkaline bismuth) diluted 40 times from
the stock solution as a section-stain\textsuperscript{12}). In some cases, unstained sections were observed.

RESULTS

Effect of fixation-methods, en bloc staining and section-staining on contrast of
fungal ultrastructure

\textit{A. alternata} spores fixed by methods a and e showed dense cytoplasm, and therefore the
contrast of cellular membranes was poor in spite of staining the sections with uranyl acetate
and lead citrate (Figs. 1 and 2). The spores fixed by methods b, c, d and f also did not show
a fine structure in the sections stained with uranyl acetate and lead citrate (Table 2). In alde-
yhyde and osmium tetroxide-fixed spores which were \textit{en bloc} stained with alkaline bismuth
(method g), the bismuth selectively reacted with the cell wall and plasma membrane. Elec-
tron-dense cell wall and plasma membrane were clearly seen even in unstained sections (Fig.
3).

Potassium permanganate-fixed (method h) or aldehyde and potassium permanganate-fixed
spores (method i) showed poor contrast of the cellular membranes in all unstained section. A
slightly increased contrast could be obtained when the sections were stained with uranyl acetate
and lead citrate (Fig. 4), and a good contrast when they were stained with alkaline bismuth solu-
tion as previously reported\textsuperscript{14}). In methods j–l, alkaline bismuth of an \textit{en bloc} stain for aldehyde
and potassium permanganate-fixed spores brought about a great increase in contrast of all cel-
lar membranes or cell wall\textsuperscript{14}). The \textit{en bloc} staining-effect was high enough in unstained sec-
tions for ultrastructural observation (Fig. 5) and also in sections stained with uranyl acetate
and lead citrate (Fig. 6).

In methods m and n, the spores fixed with aldehyde and osmium-ferrocyanide (reduced
osmium), followed by the exposure of an \textit{en bloc} uranyl acetate stain, revealed the fine cellular
methods for spores of *Alternaria alternata* in electron microscopy

<table>
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<th>ethanol (ET)</th>
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<td>4, 5</td>
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<td>(6 hr)</td>
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<td>(6 hr)</td>
<td>(4 C, 24 hr × 2)</td>
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</table>

Effect of embedding processing on resin-infiltration

A routine procedure of Spurr resin-infiltration resulted in good or medium penetration of spores fixed by methods a–g, and then a good subsequent polymerization could be reached (Table 2). Spores fixed with potassium permanganate showed poor infiltration and polymerization. The use of an *en bloc* alkaline bismuth stain for aldehyde and potassium permanganate-fixed spores (method j) also often caused poor polymerization of the resin (Fig. 5). This trouble could be considerably solved by exposing the spores to the resin for a long time (methods k and l). Preparing spores fixed with the aldehyde and reduced osmium in the routine embedding processing, very poor polymerization always occurred (method m). In this case, prior to the infiltration, additional steps were introduced: the fixed spores were dehydrated with ethanol for a long time to make spore-bound osmium sufficiently reduced. After this, the spores were heated at 60 C for 3 hr in a resin/propylene oxide mixture and then rinsed with propylene oxide (methods n–p). By passing through these processes, good infiltration was achieved (Figs. 7–9, Table 2).

**DISCUSSION**

Recently, three new types of advanced fixation methods were reported for the electron microscopic observation of biological tissues. The first type comprises of a tetra-fixation method using glutaraldehyde, osmium tetroxide and mordanting tannic acid, and an improved double fixation method using glutaraldehyde and an osmium tetroxide/potassium ferrocyanide membranes (Fig. 7). Contrary to expectation, the treatment of chitinase after the primary fixation of spores with aldehyde (method o) only increased the slight contrast of the ultrastructure (Fig. 8). Marked differences did not appear in the contrast between methods n and o. Some spores processed by method p where mordanting tannic acid was used together with reduced osmium, showed selectively electron-dense endoplasmic reticulum and nuclear membranes (Fig. 9).
Table 2. Effects of the methods for preparing Alternaria alternata spores in electron microscopy and section-staining on the ultrastructural contrast and Spurr resin-infiltration mixture. They cause the increased contrast of the cellular membranes much more than a conventional double fixation method using only glutaraldehyde and osmium tetroxide. The second type is a fixation method for certain cellular organelles and lipid droplets. They can be observed as electron-dense structures which form as the result of a markedly specific binding of osmium metals with the components of the organelles or lipid droplets. A binding can be reached by choosing the appropriate buffer. So it was found that imidazole-buffered osmium tetroxide well reacted with lipid droplets of tissues primarily fixed with glutaraldehyde, although cacodylate-buffered osmium tetroxide was poorly reactive with the lipid droplets. The zinc iodide-osmium fixative is useful in detecting RER or Golgi bodies of animal and plant cells. The third type is a fixation method using glutaraldehyde-ruthenium hexamine trichloride and osmium tetroxide-ruthenium hexamine trichloride. It is effective in preventing occurrence of cell collapse in degenerating chondrocytes. No reports, however, had been published on ultrastructures of fungal cells which were processed with the new fixation methods. In this study, therefore, these new fixation methods were for the first time applied to fungal cells. It was demonstrated that they were also useful in obtaining high contrasted images of the fungal ultrastructure. Among 16 methods applied to A. alternata spores, poor contrast was obtained in ultrastructure of the spores fixed with the double fixation methods using aldehyde and osmium tetroxide (methods a and b), aldehyde and imidazole-buffered osmium tetroxide (method e), or aldehyde and zinc iodide-osmium tetroxide (method d) or aldehyde-ruthenium hexamine trichloride and osmium tetroxide-ruthenium hexamine trichloride (method f), and the tetra-fixation method using aldehyde, osmium tetroxide and tannic acid (method c).

The fixation of the spores with potassium permanganate alone (method h) or aldehyde and potassium permanganate (method i) also gave no good results for the fine structure. However, an additional step of alkaline bismuth solution as either an en bloc stain or a section stain considerably increased the contrast of all cellular membranes and cell wall in aldehyde and potas-
sium permanganate-fixed spores (methods j-1, Table 2). This indicates that the alkaline bismuth is useful both as an *en bloc* and a section stain for aldehyde and potassium permanganate-fixed spores.

An *en bloc* alkaline bismuth stain is also useful to observe cell wall and plasma in unstained sections of aldehyde and osmium tetroxide-fixed spores (method g) because these structures were selectively stained with it (Fig. 3). Similar observations were reported in a cytochemical study of alkaline bismuth-stained sections from plant cells\(^{12}\). Alkaline bismuth solution reacts selectively with polysaccharides containing much 1,2-glycol groups of cell wall and plasma membrane in aldehyde and osmium-fixed plant cells\(^{19}\) while it reacts non-specifically with all cellular membranes in aldehyde and permanganate-fixed fungal cells\(^{42}\).

The fixation method using osmium-ferrocyanide (methods m and n) gave good results for the fine structure. The treatment of chitinase (method o) or the use of mordanting tannic acid (method p) were only slightly effective to increase the ultrastructural contrast in osmium-ferrocyanide fixed spores (Compare Fig. 7 with Figs. 8 and 9). The methods using potassium ferrocyanide were excellent for observation of the ultrastructure, especially at high magnification where they showed finer images of the cellular membranes.

In addition to problems of fixation, another difficulty for preparing *A. alternata* spores in electron microscopy exists in the resin infiltration. A low-viscosity Spurr resin is better for thick-walled spores than a high-viscosity Epon resin. Therefore, Spurr resin was certainly effective for the resin-infiltration in spores fixed with methods a–g (Table 2). On the other hand, the resin usually poorly infiltrated in potassium permanganate-fixed spores when they were prepared by the conventional infiltration procedure (methods h–j). The use of an infiltration-procedure with prolonged exposure of the potassium permanganate-fixed spores to resin could solve the problems considerably (methods k and l). Although the osmium-ferrocyanide fixative gave good contrast in ultrastructure, it was always associated with poor infiltration and polymerization of Spurr resin. In this case, the problem might have been originated by deterioration of the resin. Such a deterioration can be caused by the strong oxidizing properties of the osmium-ferrocyanide fixative against the polymerizing resin\(^{17}\). The deteriorated resin was semi-polymerized at 60°C for 3 hr in a resin/propylene oxide mixture and then removed by rinse of propylene oxide. Furthermore, the rinsed spores were processed in the infiltration procedure with prolonged exposure of the spores to the resin. This infiltration-procedure considerably eliminated the poor polymerization of resin blocks which was caused by the reduced osmium-fixative.

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**Literature cited**


和 文 摘 要

朴 俊允・大野藤吾・故 西村正啓・田辺憲太郎・甲元啓介・尾谷 浩：*Alternaria alternata* 胞子の電顕観察のための固定法と包埋法の改良

*A. alternata* 胞子をグルタルアルデヒド (GA) と四酸化オスミウム (OS) で二重固定しても電顕観察に適した細胞像は得られない。本研究では電顕標本作製が困難な本菌胞子の固定法と包埋法の改良を試みた。胞子の微細構造はアルカリベスマス (AB) 液によるブロック染色と還元オスミウム (OS-フェロシアニン化カリ混合固定液) の使用により顕著に改善された。GA と還元マンガン酸カリによって胞子を固定した後に AB ブロック染色すると、無染色切片でさえ細胞内器官の縁コントラストが増加し、十分に観察することができるようになった。また、GA と還元オスミウムで固定しさらに酢酸ウラニでブロック染色した胞子においても良好な微細構造が得られた。GA と OS によって通常通り胞子を二重固定し、さらに AB ブロック染色すると原形質膜と細胞質が特異的に染色され、この固定法がこれらの構造の観察に適していることが明らかにされた。還元オスミウムは膜系の増コントラストをもたらすが樹脂の浸透不良をつねに引き起こす。この欠点は還元オスミウムで固定した胞子を樹脂とプロピレンオキサイドの混合液で 60C、3 時間加温後、プロピレンオキサイドで洗浄し、長時間の樹脂浸透を行うことによって改善された。
Explanation of plates

Plate I

Fig. 1. A longitudinal section of an aldehyde and osmium tetroxide-fixed spore (method a). Contrast of cellular membranes is poor after section-staining with uranyl acetate and lead citrate, but the infiltration of resin is good.

Fig. 2. A section of an aldehyde and imidazole-buffered osmium tetroxide fixed spore (method e). The spore showed poor contrast of organelles after section-staining with uranyl acetate and lead citrate, and medium infiltration of resin.

Fig. 3. An unstained section of an aldehyde and osmium tetroxide-fixed spore stained en bloc with alkaline bismuth, showing a cell wall and plasma membranes of selectively high electron-density (method g). The infiltration of resin is medium.

Fig. 4. A section of an aldehyde and potassium permanganate-fixed spore (method i) showing slightly increased contrast after section-staining with uranyl acetate and lead citrate. Poor infiltration of resin is evident by the presence of many holes between the outer cell wall and the extracellular space.

Fig. 5. An unstained section of aldehyde and potassium permanganate-fixed spores stained en bloc with alkaline bismuth, showing good contrast of organelles and cell wall (method j). Many holes resulted from poor resin-infiltration were observed between extracellular space and the outermost cell wall layers.

Plate II

Fig. 6. A section of an aldehyde and potassium permanganate-fixed spore stained en bloc with alkaline bismuth (method l). Cell membranes and cell wall are highly contrasted after section-staining with uranyl acetate and lead citrate. Infiltration of resin is medium.

Fig. 7. A section of a spore fixed with aldehyde and osmium-ferrocyanide and then en bloc stained with uranyl acetate, showing the fine structure of cellular membranes after section-staining with uranyl acetate and lead citrate (method n). Infiltration of resin is medium because of once removing the deteriorated resin from spores and performing infiltration by prolonged exposure of spores to resin.

Fig. 8. A section of a spore exposed in chitinase after briefly fixing in aldehyde, fixed with osmium-ferrocyanide, and followed by en bloc uranyl acetate staining (method o). Cell membranes and cell wall are fine for observation after section-staining with uranyl acetate and lead citrate. Infiltration of resin is medium.

Fig. 9. A section of a spore tetra-fixed with aldehyde containing 0.1% tannic acid, osmium-ferrocyanide, aldehyde containing 2% tannic acid, and osmium-ferrocyanide and then stained en bloc with uranyl acetate (method p). Electron-dense endoplasmic reticulum and nuclear membranes are seen. Infiltration of resin is medium.
Plate I
Plate II