

凍結処理による *Pythium butleri* 卵胞子の採取法

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著者名	楠,幹生 一谷,多喜郎
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Preparation of Mycelium-free Oospores of *Pythium butleri* by a Freezing Method

Mikio KUSUNOKI* and Takio ICHITANI*

楠 幹生*・一谷多喜郎*: 凍結処理による *Pythium butleri* 卵胞子の採取法

Key Words: mycelium-free oospores, *Pythium butleri*.

Several methods have been used to separate oospores from mycelial fragments using different *Pythium* species¹⁾, but there have been no attempts to prepare oospores of *P. butleri*. This study was designed to compare several methods for obtaining oospores of this fungus, and especially to kill other viable propagules without destroying the oospores. Part of this work was presented at the annual meeting, Phytopathological Society of Japan²⁾.

Four isolates of *P. butleri* Subr., causing damping-off of spinach seedlings³⁾, were used. Single oospore isolates of the fungus were maintained on cornmeal agar at 15–22 C. The fungus was cultured to produce oospores for two to four weeks at 25 C in 300-ml Erlenmeyer flasks containing 50 ml of 10 % V-8 juice liquid medium supplemented with 500 ppm of wheat germ oil (Japan Impex Co., Ltd., Tokyo). Moreover, mycelial mats free from oospores were obtained in Schmitthenner's liquid medium⁴⁾ without cholesterol by culturing for 2 weeks at 25 C. The mycelial mats obtained from the above two cultures were washed twice with sterile distilled water (SDW) and homogenized by a Waring blender, except those used for freezing, and the number of oospores in each suspension was determined by a Thoma haemocytometer. The final concentration was adjusted to about 10⁵ oospores per milliliter (OPM). The homogenates were subjected to one of the treatments listed in Table 1. Freezing, however, was carried out by the method shown in Table 1, using cultures that had not been homogenized.

After each treatment, two plates containing Difco cornmeal agar supplemented with 40 ppm of streptomycin were incubated with 5 drops (about 15 mm in diam.) of the suspension per plate. The plates were examined microscopically for growth from the mycelial fragments or oospore germination every 5 hr up to 24 hr of incubation at 30 C in the dark. Some naked oospores were observed by a scanning electron microscope using the method described by Takamatsu *et al.*⁵⁾ All treatments were repeated at least twice. The data presented in this paper are typical of the experiments (Table 2).

Viable propagules other than oospore were killed completely by freezing and by

* College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan 大阪府立大学農学部

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Table 1. Treatments used for the preparation of oospore

Treatment	Description
Non-treatment	The suspension was used directly after grinding by a Waring blender ^{a)} (14,000 rpm for 2 min).
Cellulase	The suspension was mixed with an equal volume of cellulase (8% solution) ^{b)} . After 60 min of shaking at 36 C, the oospores were washed twice with SDW by centrifugation (750×g for 2 min).
Formalin	The suspension (about 3 ml) was mixed with a 1:1,000 dilution of formalin (about 40 ml) ^{c)} and incubated for about 20 min at 15 C. The oospores were washed twice with SDW by centrifugation (750×g for 2 min).
Sonication	The suspension was subjected to 40 % of maximum intensity of ultrasonic system (20 KHz) ^{d)} for 90 sec at 10–20 C.
Freezing	The culture was frozen at –20 C for 3hr and then allowed to warm (45–50 C) to room temperature. The mycelial mat was washed twice with SDW and ground in a Waring blender (14,000 rpm for 2 min).
Heating	The suspension was heated in a water bath (46 C) for 10min and cooled on ice.

a) Sakuma Seisakusho Co., Ltd., Osaka

b) "Onozuka R-10", Yakult Chemical Industrial Co., Ltd., Takarazuka, Hyogoken

c) "Formalin Special" (36 %), Wako Chemical Co., Ltd., Osaka

d) Kaijo Denki Co., Ltd., Osaka

Table 2. The effect of various treatments on growth of propagules other than oospores and oospore germination

Treatment	Growth of propagules other than oospores ^{a)}	Oospore germination
Non-treatment	+	80ab ^{b)}
Cellulase	+	93b
Formalin	–(+)	76ab
Sonication	–(+)	83ab
Freezing	–	86ab
Heating	–	59a

a) + : growth, –(+): slight growth, – : no growth

b) Within each column, entries without a common letter are significantly different (P: 0.05 by Duncan's multiple-range test).

heating (Table 2, Plate I-1) and almost completely by sonicating and by treating with formalin (Table 2). Walls of propagules other than oospore were digested partially by a cellulase, but were not killed completely (Table 2, Plate I-2, 3). Among these conditions which killed the propagules other than oospores, oospore germination was delayed by formalin treatment and sometimes depressed by heating. Sonicating and freezing treatments did not inhibit oospore germination, but the former treatment resulted in formation of about 50 % of naked oospores (Plate I-4~7).

Although sonication is used for obtaining oospore suspensions free from mycelium using *P. aphanidermatum*¹⁾, which is closely related to *P. butleri*, the freezing method

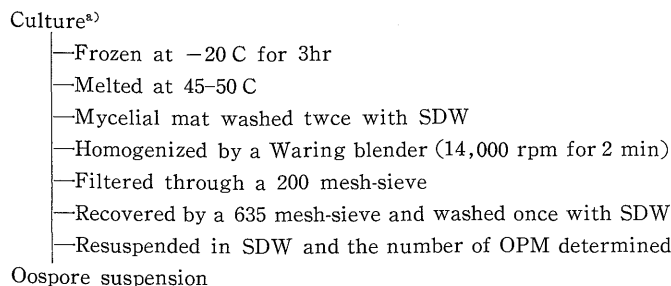


Fig. 1. Freezing method for obtaining mycelium-free oospore

- a) Cultured in 10% V-8 juice liquid medium supplemented with 500ppm of wheat germ oil for two to four weeks at 25 C in the dark.

was found to be the best among the treatments employed in the present experiment for *P. butleri*. By the present method, about 10^6 intact oospores were easily obtained from a flask (Fig. 1; Plate I-8, 9). A preliminary inoculation test was made using cucumber seedlings, and the results obtained indicated that the pathogenic ability of the oospore was retained. Oospores are the only thick-walled spherical structure in *P. butleri* and sporangia of this fungus are inflated branched filamentous³⁾. Thus sporangia of *P. butleri* can, like mycelia, be killed by freezing and oospores are the only viable propagules after freezing.

Five methods used in this experiment were also applied to *P. zingiberum* to separate oospores from mycelial fragments, but no practical method has been found so far.

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Explanation of Plate

Plate I.

1. Germination of oospores after the freezing and heating treatments ($\times c.$ 500).
Other propagules (arrows) are killed under these conditions.
- 2,3. Branching of viable hyphae (2: $\times c.$ 300) and germination of inflated sporangium (3: $\times c.$ 500) after the treatment of cellulase.
Oospores are also viable, but they do not yet germinate in this period of incubation.
- 4-7. Naked oospores resulted from sonication.
Light micrograph (4: $\times c.$ 600). Scanning electron micrographs (5: $\times c.$ 500, 6: $\times c.$ 1,600, 7: $\times c.$ 1,800).
8. Mycelium-free oospore suspension obtained by freezing method ($\times c.$ 200).
9. A high percentage of oospore germination indicating no damage in mycelium-free oospore obtained by freezing method ($\times c.$ 500).

Plate I

