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論　文（Original article）

# Mass production of conidia of *Sydowia japonica*, a candidate of male-strobilus specific biocontrol agent for preventing the pollen dispersal of *Cryptomeria japonica*

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**Abstract**

Reduction of the pollen counts of *Cryptomeria japonica* is required for control of Japanese cedar pollinosis in Japan. The fungal parasite *Sydowia japonica* is under consideration as a biocontrol agent for male strobili of *C. japonica*. This organism has been shown to be virulent only against male strobili and seems to be a potential candidate for the control of the pollen dispersal. However the fungus has never produced sufficient numbers of inoculum on the artificial solid media for the application in the field yet. Here we report a series of experiments for mass production of conidia of *S. japonica* in artificial media. No teleomorph was induced on any artificial medium but conidia were easily produced on several media. Liquid medium based on Czapek-Dox and yeast extract was most useful for the production of the conidia. Additionally, culture condition was optimized for the rapid and mass production of the conidia. This study contributed to development of the application of *S. japonica* as male strobilus-specific bioherbicide.

**Key words** : *Sydowia japonica*, *Hormonema*, *Cryptomeria japonica*, spore production, bioherbicide

## 1. Introduction

Allergy caused by pollen of *Cryptomeria japonica*, which is so called Japanese cedar pollinosis, is one of the serious allergies in Japan. Twenty five million Japanese suffer from the pollen allergy, thus effective procedure are required for the control of pollen dispersal. One of the options is breeding of cultivar of *C. japonica* lacking pollen production (Saito et al. 1998). Indeed several cultivars lacking pollen has been developed in the program of Forest agency in Ministry of Agriculture, Forestry and Fishery of Japan. However, a difficult problem about *C. japonica* trees already planted remains to be unsolved. Transplantation of all trees with pollen-lacking trees takes time, cost and efforts. Thus additional options for reduction of pollen counts should be also considered.

Recently we found a fungal parasite on the male strobili of *C. japonica*. This fungus was identified as *Leptosphaerulina japonica* in the past (Kasai 1917, Kobayashi 1970) but is now treated as *Sydowia japonica* (Hirooka et al. 2012). Most interesting feature of this fungus is that it specifically infect only to the male strobili of *C. japonica*. This feature enables us to consider the fungus as a candidate for the control of male strobili of *C. japonica*. Hirooka et al. (2013) conducted the artificial inoculation to *C. japonica* and showed that the fungus was useful for the control of the male strobili and suggested the advantages of *S. japonica* as a biocontrol agent for male strobili of *C. japonica*. However, because they use mycelial mass as inoculum and directly inoculated the fungal mycelia to each male strobilus, their preparations for inoculation experiments were laborious and time-consuming. This can be extensively improved where mass spore production and inoculation using spore spraying are possible.

Thus, the objectives of this research are as follows; 1) to evaluate a range of growth media for the production of spores, 2) to optimize culture condition for mass production of inoculum.

## 2. Materials and Methods

### 2.1 Fungal strain used in this study

The fungal strain used in this study was established from single ascospore discharged from ascocarp on the male strobilus of *C. japonica* collected at Nishi-Aizu, Fukushima, Japan in Sep 2008. Before isolation, ascocarp and ascospores were observed and confirmed its identity. The strain was maintained on potato dextrose agar plate at 20 °C until use. Also the strain was deposited as NITE P-757 and preserved in National Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE) in Japan.

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### 2.2 Growth test on agar media

Agar media used in this study are as follows: MA (Difco malt extract 20 g/l, agar 15 g/l, EBIOS yeast extract 0.5 g/l), MEA (Difco malt extract 20 g/l, Difco Bacto Peptone 1 g/l, Glucose 20 g/l, Agar 20 g/l), PDA (potato dextrose agar, Nissui), CMA (corn meal agar, Nissui), CYA (Sucrose 30 g/l, $NaNO_3$ 3 g/l, $K_2HPO_4$ 1.0 g/l, $MgSO_4$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4$ 0.01 g/l, Yeast Extract 1 g/l and Agar 15 g/l), YM (Difco malt extract 3 mg/l, Difco yeast extract 3 mg/l, glucose 10 g/l, peptone 5 g/l, agar 15 g/l), V8 ( V8 Juice 50 ml/l, agar 15 g/l, $CaCO_3$ 0.2 g/l), G25N (25 % Glycerol Nitrate Agar: $NaNO_3$ 3 g/l, $K_2HPO_4$ 1 g/l, KCl 0.5 g/l, $MgSO_4$. $7H_2O$ 0.5 g/l, $FeSO_4.7H_2O$ 0.01 g/l, Difco yeast extract 5 g/l, agar 15 g/l, pH 6.0. After agar was dissolved, 250 g glycerol to 750 g medium was added.). Mycelial plugs (5 mm diameter) were transplanted from the original isolate to each medium. The cultivation was performed in Petri dishes at 20 °C for a month for all culture media. Spore production and its morphological characteristics were examined for each medium during the incubation.

### 2.3 Growing test on liquid media

Three types of liquid media were selected in this study. They were expected to induce spore production based on the knowledge from the above growing test on agar media. They were PD (Difco potato dextrose broth 2 %), ME (Difco malt extract 2 %) and CZ (Difco Czapek-Dox broth 3.5 %, Difco yeast extract 0.15 %). Mycelial plugs (5 mm diameter) were transferred into the 100 ml of liquid media in 200 ml Erlenmeyer flasks, and were shaken at 120 rpm speed under the condition at 18 °C in the dark. After 2 weeks, morphological and cultural characteristics were examined. In addition, all of fungal cells were filtrated through the 0.22 µm nitro-cellulose membrane (Millipore, co. ltd.) and well dried at 60 °C overnight. A drop of liquid media containing fungal cells was observed with stereomicroscope (DM2500, Leica microsystems co. ltd.). Dried fungal cells were weighed.

### 2.4 Optimization of culture condition

To optimize culture condition for mass production of spores of *S. japonica*, we examined two kind of liquid media with various C/N ratios. ME and CZ liquid media were used as basal media. Each medium were emended with different volume of Difco yeast extract (0.2, 0.75, 1.5, 3, and 5 %). Different concentration of basal media (1, 2, 3, 4, 5, and 10 % for ME, 1, 2, 3.5, 5, and 7 % for CZ) emended with 1.5 % Difco yeast extract were also tested. Mycelial plugs (5 mm diameter) were transferred into the 100 ml of the liquid media in the 200 ml Erlenmeyer flasks, and shaken at 120 rpm at 18 °C in the dark. After 10 days, morphological and cultural characteristics were examined. In addition, all of fungal cells were filtrated through the 0.22 µm nitro-cellulose membrane (Millipore, co. ltd.) and well dried at 60 °C overnight. Dried fungal cells were weighed.

## 3. Results

### 3.1 Growth test on agar media

Colony appearance was different among the media used in this study (Fig.1). Although white to cream colored colony was usually developed, dark colored colony was developed only on the media containing malt extract. There

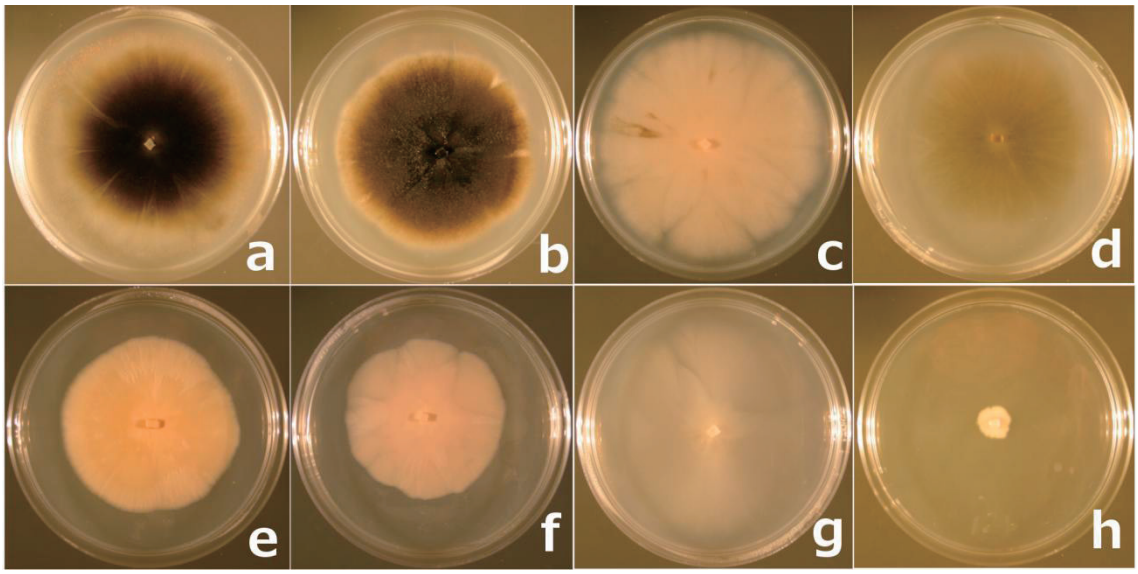


Fig. 1. Colony appearances of *Sydowia japonica* on each medium in a month.
a: MA, b: MEA, c: PDA, d: CMA, e: CYA, f: YM, g: V8, h: G25N.

are no aerial hyphae on each medium excepting MEA. Dense colony was developed on the CYA, YM and MEA media but not on the V8 and CMA. Growth rate was not different between the media excepting G25N. There was the conidial production on PDA, CYA, MA, MEA and V8 media. Conidia were produced as budding spores directly from hyphae (Fig.2).

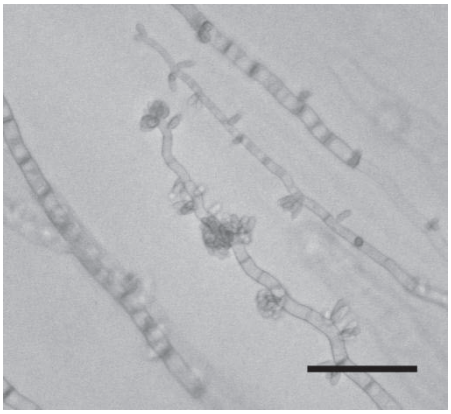

Fig. 2. Budding conidia and hyphae of *Sydowia japonica* embedding in the PDA. Scale bar = 20 µm.

### 3.2 Growing test on liquid media

Mycelial mass were developed in the two media, PD and ME, without spore production. However, in the CZ medium, conidia could be abundantly produced (Fig. 3). Budding conidia and short mycelial fragments were included in the CZ medium (Fig. 4). Each Dry weight of fungal cells on each ME, PD and CZ was 1.1, 0.7, and 1.85 mg, respectively.

### 3.3 Optimization of culture condition

In the media based on ME containing 1.5 % yeast extract, maximum dry weight of fungal cells was yielded in 5 % and 10 % of malt extract (Fig. 5A). When 5 % of yeast extract was added to 2 % malt extract, dry weight of fungal

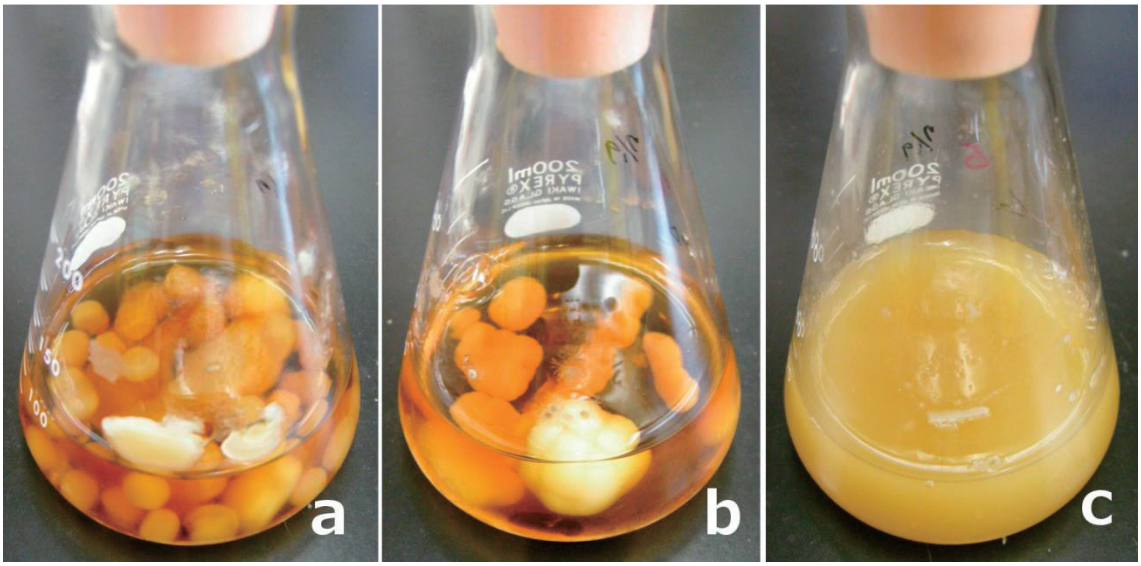


Fig. 3. Status of *Sydowia japonica* in each liquid medium. a: PD, b: ME, c: CZ, in 10 days incubation.

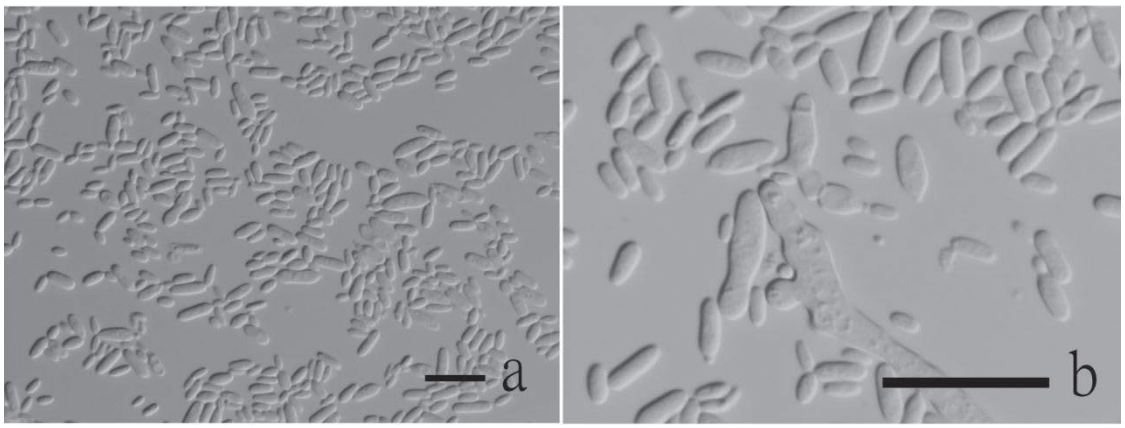


Fig. 4. Conidia and hyphae of *Sydowia japonica* in the CZ liquid media. a: conidia, b: budding fashion of conidia and hyphal fragment. Scale bar = 10 µm.

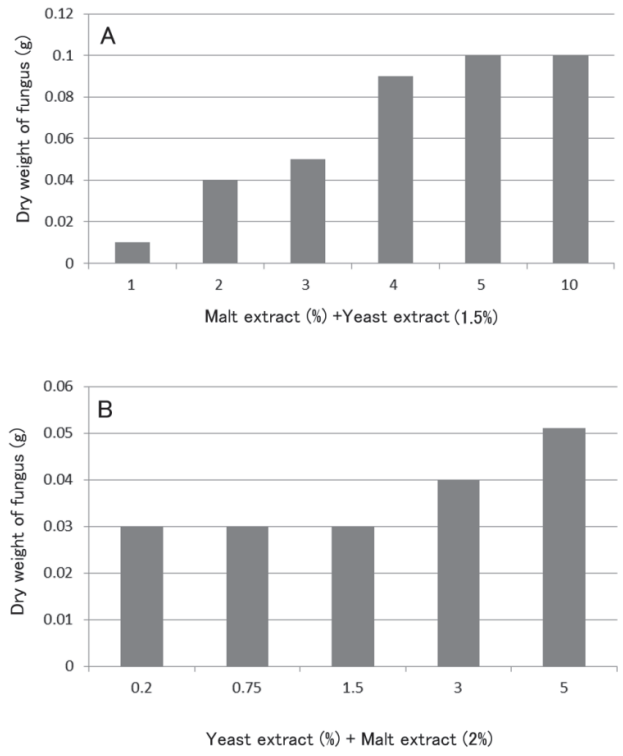


Fig. 5. Dry weights of *Sydowia japonica* grown in liquid medium based on malt extract.

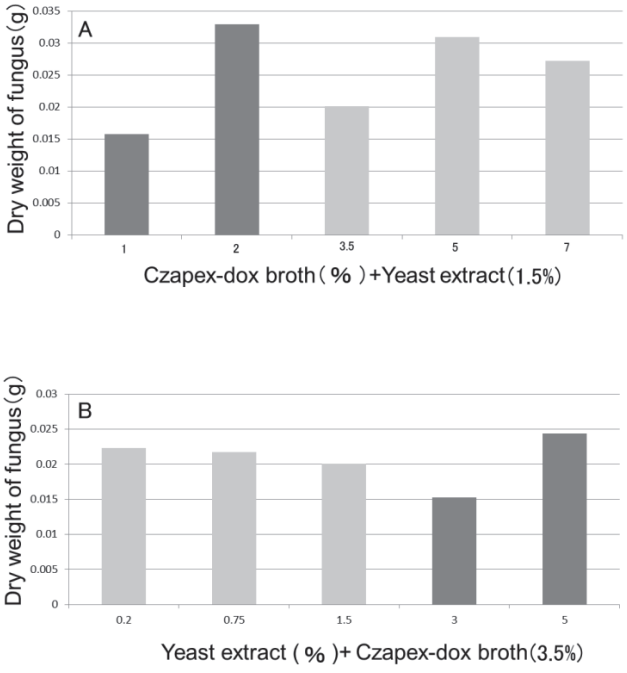


Fig. 6. Dry weights and status of *Sydowia japonica* grown in liquid medium based on Czapex-Dox broth.
Dark grey bars: Mycelial status, light grey bars: Yeast status.

cells become maximum (Fig. 5B). All of the fungal state was mycelia in the ME-based media.

In the media based on CZ containing 1.5 % yeast extract, the maximum dry weight was yielded in 2 % Czapek-Dox broth; however, it was mycelial state (Fig. 6A). When over 3.5 % of Czapek-Dox broth was added, the fungus became produced budding conidia. 5 % of Czapek-Dox broth was required for maximum yield of dried cells in the conidial state (Fig. 6A).

Where concentration of Czapek-Dox broth was fixed as 35 g/l, maximum dry weight of the fungal cells was yielded in the medium containing 50 g/l of yeast extract. However, it was mycelial state (Fig. 6B). When under 15 g/l of yeast extract was added, the fungus became produced budding conidia (Fig. 6B). There are few differences on the dry weight between the medium containing 2 g/l, 7.5 g/l and 15 g/l of yeast extract (Fig. 6B). As results, maximum dry weight of fungal cells was expected in the liquid medium containing 50 g/l of Czapek-Dox broth and 15 g/l of yeast extract.

## 4. Discussion

One of the objectives of this research was to evaluate a range of growth media for the production of spores of *Sydowia japonica*. As result, conidia could be produced in the several media. Conidia were produced as budding fashion and it was just like as *Hormonema*-like form. Although *S. japonica* is reported to have a *Hormonema*-like anamorph (Hirooka et al. 2012), we found that several media were not always useful for spore production. Difference of conidial production between media may be explained by carbon concentration and C:N ratio. Indeed, C:N ratio are known to affect spore yield and quality (Jackson & Bothast 1990, Schisler 1991). Carbon-to-nitrogen ratio of cornmeal agar which was not suitable for spore production of *S. japonica* is known to be 22:1, but those of PDA and V8 media on which conidia were produced in this study is 10:1 (Wyss et al. 2001). Thus increasing nitrate concentration is expected to increase spore production. On the basis on the result of growing test on agar media, we selected three liquid media, PD, ME and CZ for spore production and found that CZ was the most suitable medium for spore production.

To optimize culture condition for mass production of inoculum, we added different volume of carbon (Czapek-Dox broth) and nitrogen source (Difco yeast extract) to the CZ-based liquid medium. High volume of yeast extract resulted to promote mycelial state to the yeast states. This clearly suggests that *S. japonica* dimorphic changes depend on the nitrogen concentration. Such nutritional stimuli have

been extensively studied in many different fungal groups (Madhani & Fink 1998, Sanchez-Martinez & Perez-Martin 2001, Herrera & Sentandreu 2002), however, in some case, the morphogenesis such dimorphism seems to depend on more on the nitrogen source than on the N concentration (e. g. Sanna et al. 2012). Further growing tests using different nitrogen source will be also important for clarifying the mechanism of dimorphism on *S. japonica*.

The conidia in the liquid medium were produced by budding fashion and typical of *Hormonema*-type anamorph. This was also observed in the agar media, thus there seems to be no differences of conidial morph between media. However, we have to consider the quality of conidia for using as the biocontrol agent. Actually, there are reports that C:N ratio is affected not only spore yield but also germinability, pathogenicity , and virulence (Jackson & Bothast 1990, Schisler 1991). Germinability seemed not to be affected by C:N ratio of the medium, because all of conidia easily germinated and became mycelial state on PDA agar (authors, unpublished). There are known to be significant variation in protein and lipid content of spores (Jackson & Bothast 1990). Thus, we should test the pathogenicity of conidia produced in the liquid medium.

In this study, we succeeded to produce conidia in the liquid medium and established the procedure for mass spore production. The result of this study can greatly improve application of *S. japonica* as a male strobilus-specific biocontrol agent for *C. japonica*. Two ways of application can be considered on the mass conidial suspension. One is for spray application. As also Hirooka et al. (2013) suggested, conidial suspension form is most suitable for spay application, which is used for many biopesticide and bioherbicide. Other is for rapid preparation of inoculum. Only a few conidial suspensions can be used as seed for mass production of conidia in the liquid medium. In this study, mass production of conidia was achieved within only a week. Conidial suspension is not only easy for handling, preservation and useful for preparing of inoculum, but also can be applied for industrial mass production in liquid culture in large fermentation vessels which have electronic controls and monitoring. Consequently, this study highly contributes to the development of the application of *S. japonica* as a male strobilus-specific biocontrol agent.

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# スギ花粉飛散防止のための雄花特異的な生物防除資材候補、*Sydowia japonica* における分生子の大量生産

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**要 旨**

スギ花粉症抑制のためにスギ花粉量の抑制が求められている。*Sydowia japonica* はスギ雄花の特異的な生物防除資材として有望であり、スギ雄花のみに寄生性を示すことから、スギ花粉飛散抑制に有効である可能性がある。しかし本菌はこれまで人工培地上では野外散布に十分な量の接種源を生産していない。そこで本研究では人工培地上で分生子大量生産のための一連の実験を行った。使用したいずれの培地でも有性世代は形成しなかったが、いくつかの培地で分生子が形成された。ツァペック - ドクスと酵母エキスからなる液体培地が分生子形成に最も有効であった。さらに分生子の短期間かつ大量に生産するために培養条件を最適化した。本研究は *S. japonica* の雄花特異的な生物農薬としての適用技術の開発適用技術の開発に貢献する。

**キーワード：***Sydowia japonica*、*Hormonema*、スギ、分生子生産、生物農薬

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