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Labor-saving Preservation of Powdery Mildew of Strawberry by Sterilized Seedling Culture

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Abstract
Various preservation methods at low temperature have been reported for several powdery mildew fungi, but the conidia of *Podosphaera aphanis* causing powdery mildew of strawberry could not be preserved either at low temperature or by freeze-drying and liquid-drying of conidial suspensions or diseased leaf discs. Here, we developed a new technique for a labor-saving preservation of the conidia of the fungus by using the aseptic seedling culture of strawberry. Conidia produced on the seedlings could be preserved at 5°C for six months. Using this method, we could obtain the stable stock cultures of 12 strains collected from various regions in Japan.

Discipline: Plant disease
Additional key words: *Podosphaera aphanis*, latent infection, low-temperature preservation

Introduction

Powdery mildew is the most common and serious disease that affects cultivated strawberries. It is caused by the fungus *Podosphaera aphanis* (Wallr.) U. Braun & S. Takamatsu, which preferentially infects young green leaves and rapidly spreads over various parts of the plant, including the stem, buds, flowers, and fruits. Because infection, especially on fruits, markedly reduces the quality and marketable yield, immediate control of the disease is crucial.

P. aphanis is an obligate parasite that only infects living tissues of cultivated strawberry (*Fragaria* × *ananassa* Duchesne) and a few kinds of wild strawberry (*Fragaria vesca* and *Potentilla micrantha*)[1, 2, 13]. Therefore, it is impossible to cultivate the fungus artificially. Detached cotyledons, leaves, or leaf segments have probably been the most commonly used means for maintaining powdery mildew isolates in culture[9]. However, as the infectivity of *P. aphanis* is lower on the detached leaflets and leaf discs of strawberry[11], *P. aphanis* cannot survive for a long period of time on the leaf discs of strawberry. In addition, latent infection of strawberry leaf by *P. aphanis* has been reported[5]. These facts suggest that preservation using the strawberry leaf is risky, as the leaf may be infected by other *P. aphanis* isolates. For other powdery mildew fungi, various preservation methods at low temperature have been reported[3, 9, 12]. For example, *Podosphaera fusca*, a pathogen of cucurbit, could survive under low-temperature conditions by silica gel treatment[14]. However, this method has been used only for restrictive pathogens such as cucurbit. Thus, we here tested various methods to preserve *P. aphanis* on strawberry plants and developed a new technique for labor-saving preservation using sterilized seedling culture.

Materials and methods

1. Low-temperature preservation of conidia with cryoprotectant

Twelve single-conidial isolates were obtained from diseased strawberry leaves collected in Japan using Morrison's method[6] (Table 1). The conidia of *P. aphanis* (strain KT-1) were used for analyzing the effects of low-temperature preservation. Fresh conidia were suspended in 1 mL of 10% dimethyl sulfoxide (DMSO) or 10% skimmed milk as a cryoprotectant added with 1.5% sodium L-glutamate (SM) at a final concentration of 10^5 conidia/ mL. In another vial (diameter, 3 cm, and height, 6 cm), an infected strawberry leaf sample (1 cm x 1 cm) inoculated 14 days prior was embedded in 8 g

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Table 1. *Podosphaera aphanis* strains used in this study

Strain no.	Site of collection	Isolation cultivar	Date of sampling
KT-1	Kurume, Fukuoka, Japan	Toyonoka	April 5, 2000
KT-2	Kurume, Fukuoka, Japan	Toyonoka	April 5, 2000
KS-1	Kurume, Fukuoka, Japan	Sachinoka	April 5, 2000
KS-2	Kurume, Fukuoka, Japan	Sachinoka	April 16, 2000
YT-1	Miyahara, Kumamoto, Japan	Nyohou	May 8, 2000
UN-1	Utsunomiya, Tochigi, Japan	Nyohou	January 18, 2001
UN-2	Utsunomiya, Tochigi, Japan	Nyohou	January 18, 2001
ON-1	Oyama, Tochigi, Japan	Nyohou	January 18, 2001
AN-1	Ashikaga, Tochigi, Japan	Nyohou	January 18, 2001
SN-1	Souma, Fukushima, Japan	Nyohou	January 19, 2001
NN-1	Natori, Miyagi, Japan	Nyohou	January 20, 2001
WT-1	Watari, Miyagi, Japan	Toyonoka	January 20, 2001

All strains were maintained using the method developed in this study.

of non-sterile agar powder (Wako Pure Chemical Industries, Ltd., Osaka, Japan) . Each sample was kept at 0, -20, −40, or −80°C for 24 h after samples were subjected to pre-cooling treatment at 0, 4, or 10°C for 2 h. After preservation, the DMSO and SM samples were rapidly thawed in a water bath at 50°C for 2 min and transferred directly onto water agar (WA, 2% agar) by brush. The plates were incubated at 20°C for 24 h, and the germination rate was determined. A total of 300 conidia were investigated for each experiment. Each data set represents the mean of three replications.

2. Liquid nitrogen preservation of conidia

Ten infected strawberry leaf samples (1 cm x 1cm) were kept at 25°C for 6 and 12 h in a desiccator, in which a beaker containing 100 ml of a $MgCl_2$•$6H_2O$ saturated solution was placed to maintain 33% relative humidity. Each leaf disc was placed in a cryogenic tube (2-mL serum tube; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and pre-frozen for 1 h each, serially, at 0°C, −20°C, and −80°C, and then finally frozen at −165°C conserved in the vapor phase of liquid nitrogen. After the frozen leaves were kept at −165°C for 24 h, they were inoculated on WA plates and incubated at 20°C for 24 h for determination of the germination rate. A total of 300 conidia were investigated. Each data set represented the mean of three replications.

3. Preservation of conidia by freeze-drying and liquid-drying

The fresh conidia, which were suspended in DMSO or SM at a final concentration of 10^5conidia/mL, or the leaf discs embedded in agar powder kept at −20°C for 24 h were freeze-dried or liquid-dried using a vacuum dryer (Taite VD-400F; Taitec Co., Saitama, Japan). After vacuum-drying for 24 h, the conidia were re-suspended in 0.5 mL of sterilized water, and the germination rate was determined as described above.

Table 2. Number of leaf discs latently infected with *Podosphaera aphanis*

Cultivar	Date of sampling	No. of diseased leaf discs [a] (per 400 discs)
Toyonoka	April 8, 1999	3
Toyonoka	April 8, 1999	3
Toyonoka	April 10, 1999	0
Sachinoka	April 12, 1999	1
Sachinoka	April 12, 1999	0

[a] Healthy leaf disks were kept at 20°C for 10 days and the number of discs showing visible symptoms were counted.

4. Confirmation of latent infection of healthy leaves

Four-hundred leaf discs (diameter, 2 cm) were obtained from apparently healthy strawberry leaves collected in five disease-free green houses at Kurume, Fukuoka, Japan (Table 2). Eight leaf segments were placed abaxial side up in Petri dishes (diameter, 9 cm) lined with wet filter paper. Fifty Petri dishes, with a total of 400 leaf segments, were used in this experiment. Visible symptoms were investigated after a 10-day treat-

ment under a 12-h light/12-h dark cycle at 20°C.

5. Preservation of conidia on aseptic seedlings culture

Fully mature strawberry fruits (cultivar, Toyonoka) (600 g) were mashed in 500 mL of distilled water in a juicer (MX-M2; National House Industrial Co., Osaka, Japan), and the juice was filtered using gauze to collect the strawberry seeds. Seeds were completely dried in a clean bench. Then, the seeds (20 g) were immersed in 50 mL of 100% H_2SO_4 for 5 min and stirred by glass rod to remove the seed coat. The seeds were washed in flowing water for 1 h and rinsed using 70% ethyl alcohol to obtain sterilized seeds. Twenty seeds obtained in this manner were sown in a polycarbonate pot (CB-1B; AS ONE Co., Osaka, Japan: diameter, 80 mm and height, 102 mm) containing 50 g of autoclaved soil, and then the pot was completely sealed by cap to avoid contamination and kept at 20°C in a clean room. The germination rate of strawberry seeds treated with 100% H_2SO_4 solution was higher than that of non-treated seeds, as previously reported[13].

The conidia spread on WA plate were subjected to isolation of a single conidium (strain KT-1) under a stereoscopic microscope. Each single conidium was inoculated on a first true leaf by needle 20 days after sowing. The inoculated place on the leaf was marked with a felt-tipped marker.

Table 1 lists 12 strains of powdery mildew isolated from strawberry plants collected from various regions of Japan. The strawberry seedlings bearing visible lesions as a result of the inoculation were preserved in the room regulated at 5°C under 12-h light/day conditions (Fig. 2). Illumination was maintained using a plant-growing fluorescent light (plant FL40S•BRN; Toshiba, Tokyo, Japan). Light is placed at about 20 cm above the surface of strawberry. After the infected seedlings were preserved for six months, the seedlings were transferred to a room (20°C) and incubated for seven days. The fresh conidia produced were used for inoculation on healthy strawberry leaves by the dusting method. The growth of *P. aphanis* on strawberry leaves was observed for 10 days after inoculation. Other isolates (11 isolates) collected from various region of Japan were also examined for re-inoculation.

Results and discussion

1. Effects of low-temperature preservation on *P. aphanis*

We used the low-temperature preservation method for powdery mildew reported by Ozaki, Takahashi, and Sirakawa[12], i.e., the conidia were suspended in DMSO or SM and frozen. However, no germination was observed after 24 h of low-temperature preservation treatment in either DMSO or SM (Table 3). We also attempted to preserve conidia by the freeze-drying and liquid-drying methods, but were unsuccessful. The conidia transferred to WA from liquid nitrogen did not germinate. The result suggests that *P. aphanis* cannot be preserved in liquid nitrogen, although the fungus causing powdery mildew of cucurbits can be preserved in liquid nitrogen[10].

Table 3. Germination rate (%) of conidia preserved for 24 h at low temperature

Preservatives	Temperature			
	0°C	−20°C	−40°C	−80°C
DMSO[a)]	0	0	0	0
S M[b)]	0	0	0	0
Agar powder[c)]	22.3	0	0	0

a) Conidia were suspended in 10% dimethyl sulfoxide and frozen.
b) Conidia were suspended in skim milk (10% SM added with 1.5% sodium-l-glutamate) and frozen.
c) Conidia were embedded in 8 g of non-sterile agar powder and frozen.

One of the most popular methods for the long-term preservation of the fungi causing powdery mildew is to use cleistothecia, which are small saclike bodies containing ascospores. Dried cleistothecia obtained from *Blumeria graminis* f.sp. *hordei* have been reported to be preserved for 13 years[7]. *P. aphanis* causing powdery mildew in strawberry plant has been reported to be heterothallic, and the formation of cleistothecia has been reported overseas[4]. However, we did not find cleistothecia of *P. aphanis* on strawberry plants in Japan, as Nakazawa and Uchida[8] did. Thus, we considered it difficult to use this method for the long-term preservation of the conidia of *P. aphanis*.

P. aphanis causing powdery mildew in cucurbits has been reported to be successfully preserved for 320 days at −20°C using the agar-embedding method[12]. However, this method was also ineffective for the preservation of the conidia of *P. aphanis* in this experiment: i.e., the germination rate of the conidia was only 22.3% at 0°C for 24 h and reduced to 0% after a 10-day preservation period (Table 3). In addition, no germination was observed for 24 h at −20°C, −40°C, or −80°C. These results suggest that low temperature can be assumed to not be preferable for preservation of *P. aphanis*.

Fig. 1. Symptom on seedlings grown from sterilized seeds by inoculation with conidium of powdery mildew
Symptom appeared 14 days after inoculation at 20°C

Fig. 2. Seedlings of strawberry inoculated with *Podosphaera aphanis* and preserved at 5°C under light

2. Preservation of *P. aphanis* using sterilized seedlings grown from sterilized seed

When a single conidium was inoculated on a first young leaf of a seedling, many colonies of the fungus causing powdery mildew in strawberry rapidly appeared on the upper surface of the leaf after 10 days (Fig. 1), unlike in mature plants that normally show symptoms on the lower leaf surface.

The fresh conidia produced on the seedlings preserved at 5°C for six months successfully produced on strawberry leaves (Fig. 2). The resultant conidia had the ability to cause powdery mildew on other strawberry leaves. The subculture could be maintained for eight months in this experiment. In addition, the subculture could be maintained at least for two years by transferring it to new strawberry pots every six months.

All isolates (12 isolates) collected from various regions of Japan also formed conidia under the same conditions, and the resultant conidia showed infectivity. These results indicate that the sterile seedling method developed in this study could be a simple and convenient method as a labor-saving method for the preservation and preparation of stable stock of *P. aphanis*.

In confirming the latent infection of healthy leaves, a few leaves showed typical symptoms after a 10-day incubation period at 20°C (Table 2). This indicates that naturally intact leaves are insufficient for stock cultures of mildew because contamination with other mildew strains often occurs. For Rosaceae belonging to strawberry, the latent infection by *Sphaerotheca pannosa*[16] and *Podosphaera clandestina*[17] have been reported on rose and hawthorn, respectively. It is important to use seedlings from sterilized seeds as described in this paper to prevent contamination with other powdery mildew strains. Because this method can be used with ease in a low-temperature room with light, it should be applicable as a simple technique for preservation of powdery mildew of strawberry.

References

1. Abiko, K. (1982) Specialization of parasitism of *Shaerotheca humuli* (DC.) Burr. Bull. Veg. & Ornam. Crops Res. Stn. Japan, Ser. A.10, 75−79 [In Japanese with English summary].
2. Arsic, M. (1965) Proučavanje biologije i suzbijanja parazita jagode *Sphaerotheca macularis* (Wallr.) Jaczewski. *Zastita bilja*, **84**, 116−170.
3. Dahmen, H. Staub, T. & Schwinn, F. J. (1983) Technique for long-term preservation of phytopathogenic fungi in liquid nitrogen. *Phytopathol*. **73**, 241−246.
4. Gadoury, M. D. et al. (2010) Initiation, development, and survival of cleistothecia of *Podosphaera aphanis* and their role in the epidemiology of strawberry powdery mildew. *Phytopathol*. **100**, 246−251.
5. Miller, T. C. et al. (2003) Effects of Temperature and Water Vapor Pressure on Conidial Germination and Lesion Expansion of *Sphaerotheca macularis* f. sp. *fragariae*. *Plant Dis*., **87**, 484−492.
6. Morrison, R. M. (1960) Studies of clonal isolates of *Erysiphe cichoracearum* on leaf disk culture. *Mycologia*, **52**, 388−393.
7. Moseman, J. G. & Powers, H. R. Jr. (1957) Function and longevity of cleistothecia of *Erysiphe graminis* f. sp. *hordei*. *Phytopathol*. **47**, 53−56.
8. Nakazawa, Y. & Uchida, K. (1998) First Record of Cleistothecial Stage of Powdery Mildew Fungus on Strawberry in Japan. *Ann. Phytopathol. Soc. Jpn*., **64**, 121−124.

9. Nicit, P. C., Bardin, M. & Dic, A. J. (2002) Basic methods for epidemiological studies of powdery mildews: culture and preservation of isolates, production and delivery of inoculum, and disease assessment. *In* The powdery mildews, eds. Bélanger, R. R. et al., APS Press, St. Paul, MN, 83−99.
10. O'Brien, R. G. & Weinert M. (1994) A storage technique for cucurbit powdery mildew (*Sphaerotheca fuliginea*). Australas. *Plant Pathol*., **23**, 86−87.
11. Okayama, K. et al. (1995) A Simple and reliable method for evaluating the effectiveness of fungicides for control of powdery mildew (*Sphaerotheca macularis*) on strawberry. *Ann. Phytopathol. Soc. Jpn*., **61**, 534−540.
12. Ozaki, K., Takahashi K. & Shirakawa, T. (1995) Long-Term Preservation of *Sphaerotheca fuliginea* by Freezing. Ann. Rep. Soc. Plant Protect North Japan. 46, 88−90 [In Japanese with English summary].
13. Peries, O. S. (1962) Studies on strawberry mildew, caused by *Sphaerotheca macularis* (Wallr. ex Fries) Jaczewski. *Ann. appl. Biol*., **50**, 211−224.
14. Pe´rez-Garc´ıa1, A. et al. (2006) Long-term Preservation of *Podosphaera fusca* Using Silica Gel. *J. Phytopathol*. **154**, 190−192.
15. Scott, D. H. & Ink, D. P. (1945) Germination of strawberry seed as affected by scarification treatment with sulphuric acid. *Proc. Amer. Soc. Hort. Sci*., **51**, 299−300.
16. Xu, X.-M. (1999) Effects of temperature on the latent period of the rose powdery mildew pathogen, *Sphaerotheca pannosa*. *Plant Pathol*., **48**, 662−667.
17. Xu, X.-M. & Robinson, J. D. (2000) Effects of temperature on the incubation and latent periods of hawthorn powdery mildew (*Podosphaera clandestina*). *Plant Pathol*., **49**, 791−797.