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NOTE

A Simple and Reliable Method for Obtaining *Entomosporium* Monoconidial Isolates

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Monoconidial isolates of *Entomosporium mespili* were successfully cultured using a simple isolation procedure. A detailed description of the steps required for isolating *E. mespili* is provided. The characteristic pattern of conidial germination and growth on potato dextrose agar is also described. The process that was successful in obtaining pure isolates involved collecting material in the morning, picking a glistening white mass of conidia under dissecting microscope magnification, depositing a mass of conidia onto a drop of water on a glass slide, streaking the conidial mass onto potato dextrose agar, incubating the plate for 48 h, and transferring the characteristic fan-shaped colonies to fresh medium.

***Keywords*:** *Entomosporium mespili*, fan-shaped colony, germination, isolation technique

Although the most defining characteristic of Ascomycete fungi is the formation of sexually produced sac-like structures called asci, which usually contain eight ascospores, this family of fungi also depends on asexual reproductive cycles to proliferate. It is common for Ascomycete fungi to have different names for both of their sexual (teleomorphic) and asexual (anamorphic) states. This will occur when the teleomorphic and anamorphic states of a pathogen are identified separately.

The genus *Entomosporium* contains a single species, *E. mespili* (DC.) Sacc., which has been identified in its teleomorphic state as *Diplocarpon mespili* (Sorauer) B. Sutton (Nag Raj, 1993). In its anamorphic state it forms fruiting bodies called acervuli (Sutton, 1980; Sinclair *et al*., 1987). Characteristic symptoms of acervuli damage include leaf and fruit spots on woody ornamentals in the Rosaceae family, which includes such genera as *Amelanchier*, *Aronia*, *Crataegus*, *Cydonia*, *Eriobotrya*, *Heteromeles*, *Malus*, *Mespilus*, *Photinia*, *Pyracantha*, *Pyrus*, *Raphiolepis*, *Sorbus*, and others (Sivanesan and Gibson, 1976). Aside from extensive studies performed on the growth and sporulation of this species (van der Zwet and Stroo, 1985), very little research has focused on the *in vitro* culture of *E. mespili*, despite the obvious economic importance of the host plants. Indexing of culture collections from the World Federation for Culture Collection (http://www.wfcc.info/) has shown that only four living cultures of *E. mespili* have been deposited in the NITE Biological Resource Center (NBRC 9932, 9933, 30202, and 33221).

It is reasonable to believe that only a few pure cultures of *E. mespili* have been collected to date because of the difficulty in obtaining monoconidial isolates. Moreover, this fungus grows extremely slowly, exhibiting a maximum growth rate of 2.5 mm per week over a 2-month period on potato dextrose agar (PDA, Difco) (Stowell and Backus, 1966). Stathis and Plakidas (1959) described one method for obtaining monoconidial isolates of *E. maculatum* (syn. *E. mespili*); however, this method was time-consuming and unreliable. For instance, when the conidial mass was spread on media, isolates became contaminated by other faster growing fungi or bacteria.

This paper describes a more reliable procedure that can be used to obtain monoconidial isolates of *E. mespili*. Leaves of *Amelanchier asiatica* heavily infected with *E. mespili* were collected in the morning (around 9 a.m.) and afternoon (around 4 p.m.) on June 13, 2006. Samples collected on cloudy days provided the best results. Upon collection from the plant, each sample was placed into a plastic bag for transfer to the laboratory. Within 3 h from the time of collection, the success of two different methods for preparing a conidial suspension was analyzed. In the first isolation method, a single acervulus with or without host tissue was carefully cut from the leaf using a sterilized scalpel. Each acervulus was gently shaken onto a drop of sterile water on a glass slide using sterile forceps. The second isolation method involved picking a mass of conidia with a scalpel tip from an acervulus on the leaf and then placing it into a drop of sterile water on a glass slide. It was later determined that the second isolation technique produced a less contaminated sample. Thus, it was selected for use in this study.

The *E. mespili* conidial suspension prepared on the glass slide was transferred and then spread onto PDA, PDA with 200 mg/L of streptomycin·HCl, and water agar using a sterilized wire loop. After inoculation, the plates were incubated

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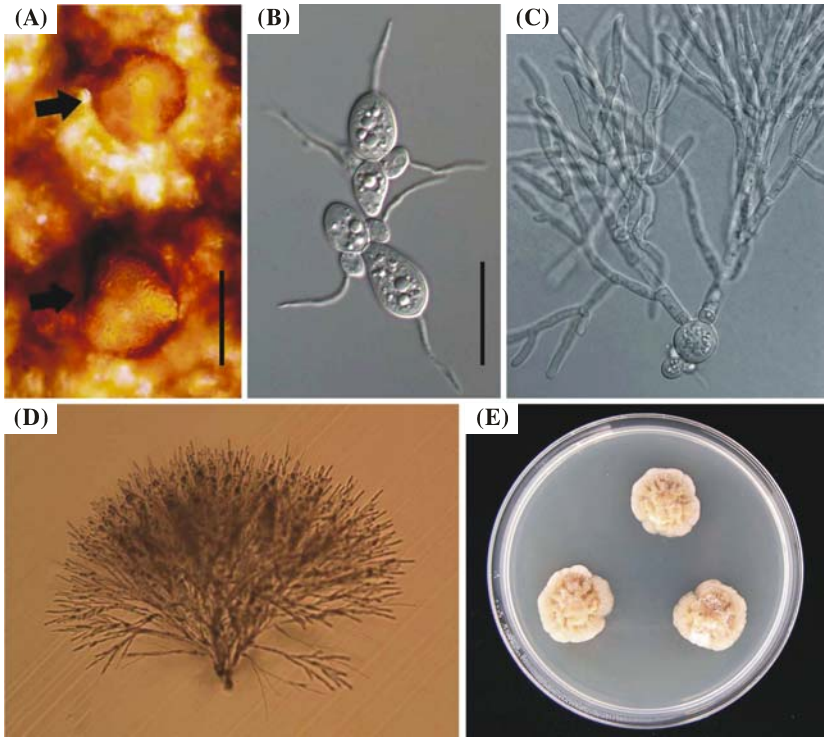

Fig. 1. Description of *Entomosporium mespili*. (A) Acervuli formed on the upper leaf surface of *Amelanchier asiatica.* Note the glistening mass of conidia in the acervuli (arrows). Bar=200 μm. (B) Conidia. Bar=20 μm. (C) Germination of a conidium, forming fan-shaped growth 3 days after incubation. (D) Characteristic fan-shaped growth of a young colony 7 days after incubation. (E) Two-month-old colonies on a PDA.

at room temperature under dark and dark/light conditions. Each plate was examined for conidial germination under a dissecting microscope every 24 h for 3 days. Each colony was transferred to a new PDA plate using a sterilized needle and then incubated at 25°C to promote growth.

E. mespili conidia (Fig. 1B) were easily identified for isolation because they typically germinated in one direction (Fig. 1C), forming fan-shaped colonies (Fig. 1D). Germ tubes were typically produced from basal cells of the conidia (Fig. 1C). To our knowledge, this is the first report on the characteristic pattern of conidial germination in *Entomosporium* species.

Infected leaves collected early in the morning when humidity levels were relatively high seemed to produce an abundant number of viable conidia. Acervuli centrally located on the lesions of the infected leaves glistened in the light of the dissecting microscope (Fig. 1A). This suggests the presence of fluid that would support the viability of conidia formed within these acervuli. Acervuli on infected leaves collected in the afternoon did not glisten when examined using the dissecting microscope. Desiccation may have taken place, leading to the reduced viability of conidia. If samples are to be collected in the afternoon or during dry periods, it is recommended that the specimens be placed in a plastic bag overnight before examining under a dissecting microscope. Masses of conidia in the acervuli that did not glisten remained as they were. Acervuli that were considered immature (i.e. covered with unruptured host epidermis) ultimately glistened and exuded conidia. Although there were relatively few acervuli exhibiting mass conidia exudation, there were enough to obtain monoconidial isolates. These acervuli were determined to be the best for use in this isolation technique.

There was no difference between the success of germination on PDA, PDA with streptomycin, and water agar. Moreover, there was no difference between germination success for plates treated with dark and dark/light conditions. The characteristic fan-shaped colony of germinated conidia (Fig. 1D) was more visible on PDA than in case of water agar. This was due to semitransparent property of PDA, therefore, PDA is recommended over the other types of plating material. The addition of streptomycin to the PDA media should help suppress bacterial contamination; however, based on data from this study, it was not necessary, since most plates were free from saprophytic bacteria contamination.

Once monoconidial isolates of *E. mespili* are obtained, purity confirmation is necessary. Production of conidia was visible about 6 weeks after isolation. Figure 1E shows 2-month-old colonies of isolate KACC42436. The addition of thiamine to PDA to enhance sporulation is considered beneficial for this process (van der Zwet and Stroo, 1985). Since the temperature and pH range for growing *E. mespili* are not critical (Horie and Kobayashi, 1979), normal PDA at room temperature is sufficient for the isolation process.

Stathis and Plakidas (1959) described a method for obtaining single spore cultures. This method was time-consuming, requiring at least 2 weeks to find colonies for isolation, and produced a large number of plates contaminated with sapro-

phytic fungi and bacteria. The senior author of the present study applied this method in 2005 as a test; all six plates were contaminated with saprophytic fungi (unpublished data). The primary advantage of the isolation procedure outlined in this paper is the early detection of colonies and, thus, easy escape from possible contaminants. This is accomplished by separating the conidia of *E. mespili* from other saprophytic fungi under a dissecting microscope 2-3 days after plate inoculation. The specimens used in this study have been preserved at Korea University with accession number KUS-F21854. The culture ex KUS-F21854 has been deposited in Korea Agricultural Culture Collection under accession number KACC42436.

Based on the above-mentioned experience, we describe a simple and reliable method for obtaining monoconidial isolates of *E. mespili* as follows:

1. Pick a mass of conidia with a scalpel tip from an acervular conidioma (Fig. 1A) formed on the surface of leaf lesion under a dissecting microscope.
2. Put the conidial mass onto a drop of sterilized water on a glass slide (simply cleaned, but usually not necessary to be sterilized). Dispersal of the conidia in water can be visualized using a dissecting microscope by adjusting the mirror beneath the stage.
3. Streak a loopful of spore suspension onto plates of PDA and incubate at 25°C for 2-3 days.
4. Examine the plates under a dissecting microscope to locate the germinating conidia showing characteristic fan-shaped growth (Figs. 1C and D).
5. Transfer the germinating conidia to new PDA plates.

The above procedure was developed in 2006 and has been used successfully in the mycology lab of Korea University. Recently, damage of Entomosporium leaf spots in commercial fields of *Photinia glabra* in Jeju, Korea, was reported (Seo *et al*., 2010). An epidemic of Entomosporium leaf spots in the nursery of *Eriobotrya japonica* caused severe economic losses during 2008-2010 in the southern part of Korea (Seo *et al*., unpublished data). Dr. S.T. Seo of Korea Forest Research Institute failed to culture the pathogens from the infected leaves of two host plant species in 2008. However, in 2009 and 2010, our procedure was successfully used to obtain monoconidial isolates from the two leaf spot diseases. These events urged us to publish our simple method.

The general scheme of the above procedure, commonly called 'streaking method', is not novel but has been used to obtain monoconidial isolates among diverse fungi (Dhingra and Sinclair, 1995). However, it is difficult to adopt this method for isolating slow-growing fungi due to faster-growing contaminants. If the early detection of young colonies on the plates, before being overgrown by contaminants, is possible, this method can be applied for other slow-growing acervuli-forming fungi like *Marssonina* spp.

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