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An optimized protocol of single spore isolation for fungi

*Ke ZHANG, Yuan-Ying SU & Lei CAI**

State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P. R. China

Abstract – Pure culture is important for taxonomic, physiological and various application studies of fungi. Many protocols for obtaining pure cultures have been reported, of which single spore isolation was used most frequently in mycological studies. However, previously reported methods were relatively complicated and time consuming. In this paper, an optimized protocol of single spore isolation is introduced. Compared to previous protocols, this protocol is favorable in terms of its lower contamination rate, is less time consuming, and requires only simplified procedures with common and easily accessible apparatus.

Mycological techniques / phytopathogens / Pure culture / strain

INTRODUCTION

Obtaining pure cultures is essential in mycological studies. In phytopathology, surface sterilization and single spore isolation are the two most frequently used isolation strategies to obtain pure cultures (Hawksworth, 1974). The "surface sterilization" method is simple and convenient. However, when the plant materials are infected by more than one fungal species, the fast growing species often suppress the others during isolation procedures. In addition, endophytes are also frequently isolated together with targeted pathogenic fungi. For example, *P. capitalensis*, a widely distributed endophyte, often co-occurs with phytopathogens (Baayen *et al.*, 2002, Glienke *et al.*, 2011, Wong *et al.*, 2012).

Several single spore isolation methods have been introduced. Booth (1971) recorded several methods of single spore isolation: single spores could be obtained either by moving single spores with a capillary pipette from a spore suspension onto a glass slide, or by directly picking single spores using microscopic observation. Using these protocols with success is highly dependent on the skill of the operators. Choi *et al.* (1999) and Goh (1999) described more practical protocols of single spore isolation, which were subsequently adopted in many studies (Cai *et al.*, 2004; Wikee *et al.*, 2011; Liu *et al.*, 2011; Hu *et al.*, 2012). However, these methods are relatively complicated and time-consuming, and are often subject to serious contamination. The contamination rate highly depends on the skill of the operator and the cleanness of the working environment. Various bacteria, yeasts and filamentous fungi are frequent contaminators during the operation. The contamination by bacteria can be avoided by adding antibiotics into the media, and yeast contamination can be minimized by diluting the spore suspensions (Choi *et al.*, 1999; Goh, 1999). Contamination by filamentous fungi, on the other hand, especially the more common species of *Aspergillus* or *Penicillium* is difficult to be avoided. Spores of these fungi are usually from air circulation and surface of plant materials (Choi *et al.*, 1999; Goh, 1999).

* Corresponding author: mrcailei@gmail.com

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In this paper, a more reliable and efficient protocol for single spore isolation is introduced. The new protocol is less time consuming and easier to operate. An experimental comparision between the new protocol and that from Choi *et al.* (1999) was conducted. The results show that the optimized protocol is remarkably efficient in decreasing the contamination rate.

MATERIALS AND METHODS

Plant material and apparatus: For the comparison between techniques, diseased plant leaves of *Mahonia fortunei* (collected from Changning District, Shanghai, China) containing well-developed pycnidia were used as the test materials. The pathogenic fungus on the leaves was identified as *Phyllosticta concentrica* using morphological characters.

Technical equipment needed in this protocol include a micropipette and tips, a syringe (or glass needle), extra fine tweezers, an alcohol lamp, petri dishes (90 mm and 60 mm), centrifugal tubes, votex in Fig. 1, as well as a dissecting microscope and a laminar flow cabinet that are not shown in the figure.

Preparation: Materials and technical equipment should be sterilized in advance, including culture media, distilled water, pipet tips, and petri dishes. Sterilized distilled water (200 µL) is transferred into several sterilized centrifugal tubes. Sterilized petri dishes (90 mm) containing 2-4 mm thick 10% strength potato dextrose agar (PDA) with 50 µg/mL antibiotics (penicillin or streptomycin) are prepared for isolation and culturing. Depending on the germination ability of the fungal spores, other alternative media such as water-agar (WA) and PDA could be used. Squares (ca. 10×10 mm) are marked on the reverse sides of petri dishes to help with locating single spores.

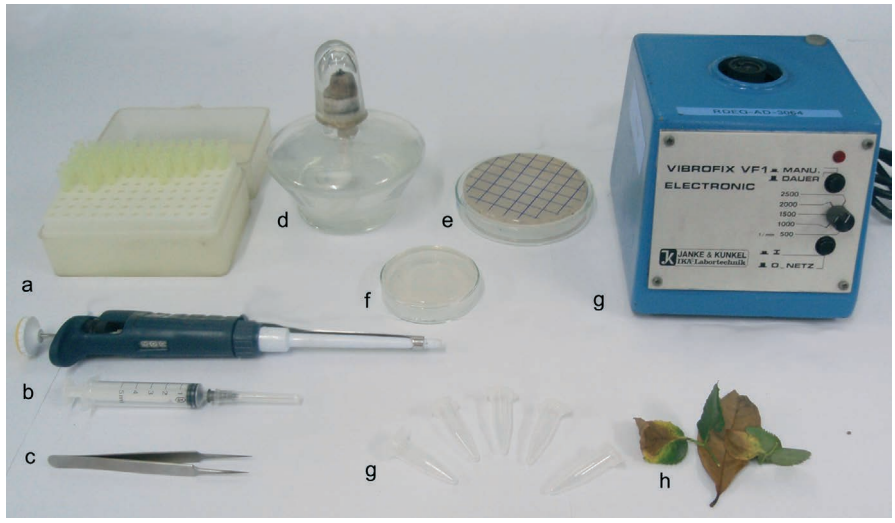

Fig. 1. Materials and apparatus used in the isolation. **a.** Micropipette and pipet tips. **b.** Syringe. **c.** Extra fine tweezers. **d.** Alcohol lamp. **e.** 90 mm petri dish with marked squares. **f.** 60 mm petri dish. **g.** Centrifugal tubes and vortex. **h.** Plant materials.

Fig. 2. Using extra fine forceps to pick up fruit body.

Spore suspension: To clean the work area, 75% ethanol was used to wipe the workbench and the dissecting microscope. An alcohol lamp is ignited beside the dissecting microscope, and air currents should be reduced to a minimum.

The plant material is gently surface sterilized by buffer with 75% ethanol and then examined using a dissecting microscope. Fruit bodies are picked out close to a flame, crushed into pieces using extra fine forceps (Fig. 2), and transferred into the sterilized water in the centrifugal tube. The centrifugal tube is then covered quickly and stirred with a vortex to obtain a homogeneous spore suspension (Figs 3-4).

The 200 µL spore suspension in the centrifugal tubes is then transferred onto the media plate by micropipette in laminar flow cabinet, with one single drop in each marked square (Fig. 5). Then petri dishes are sealed with parafilm and incubated under room temperature (ca. 25°C). The incubation time depends on the germination features of different fungi. Easily germinating species are suggested to be examined after 6 hours' incubation, while the conidia of some slow growing fungi such as *Phyllosticta* species won't germinate within the first 1-2 days (Shaw *et al.*, 1998).

Single spore isolation: The working area should be sterilized with 75% ethanol before the examination of spore germination. Two alcohol lamps are recommended to be used besides the dissecting microscope, and the air current should be controlled to minimum. Adjust the focus point of the dissecting microscope to the surface of the media to find germinating spores (Fig. 6). A small piece of media with the target spore attached should be picked up using a sterilized syringe or glass needle, and transferred onto a 60 mm media plate (or 4-5 media pieces on one 90 mm media plate evenly). At least 10 spores should be transferred and cultured under room temperature (ca. 25°C) to get pure colonies.

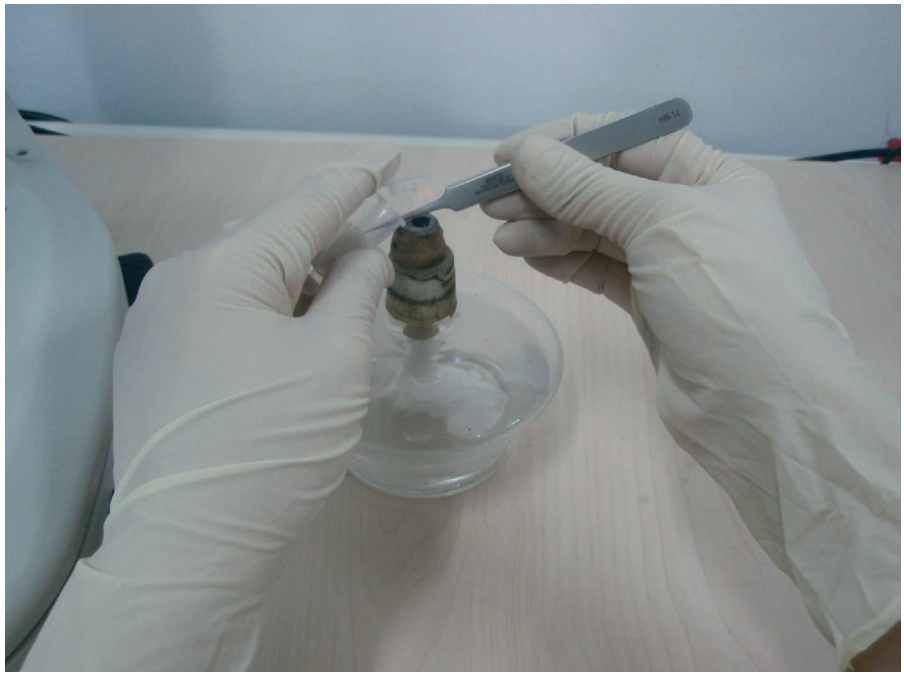

Fig. 3. Transferring crushed fruit body into sterilized water in centrifugal tube.

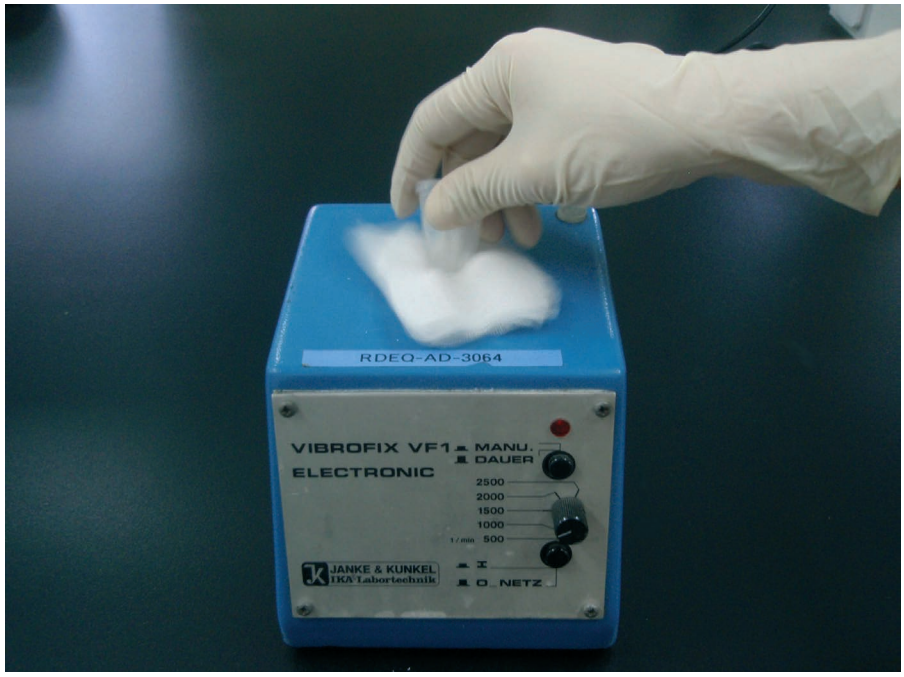

Fig. 4. Stirring suspension in centrifugal tubes using vortex.

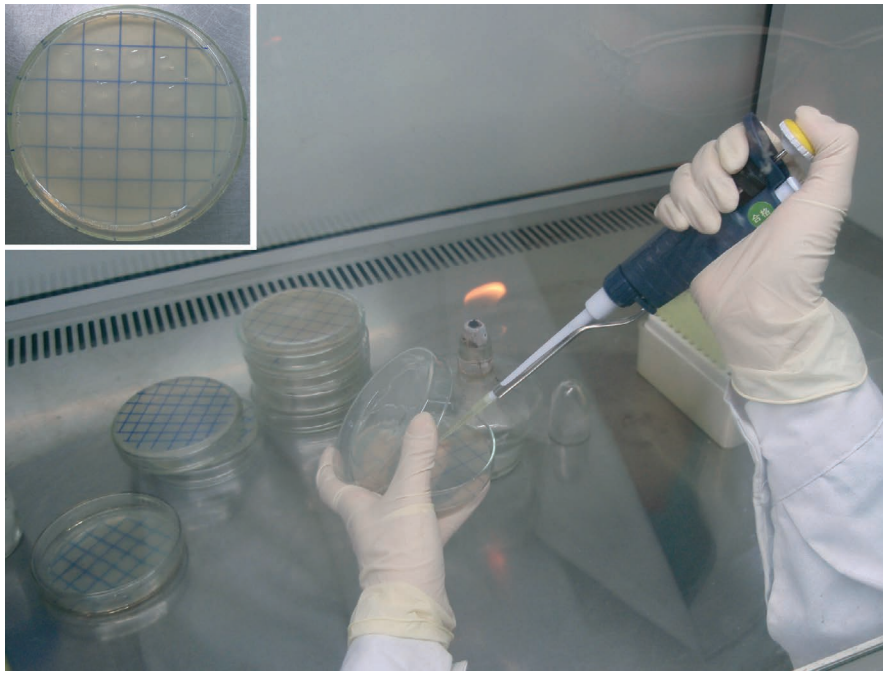

Fig. 5. Using micropipette to transfer suspensions onto agar plates.

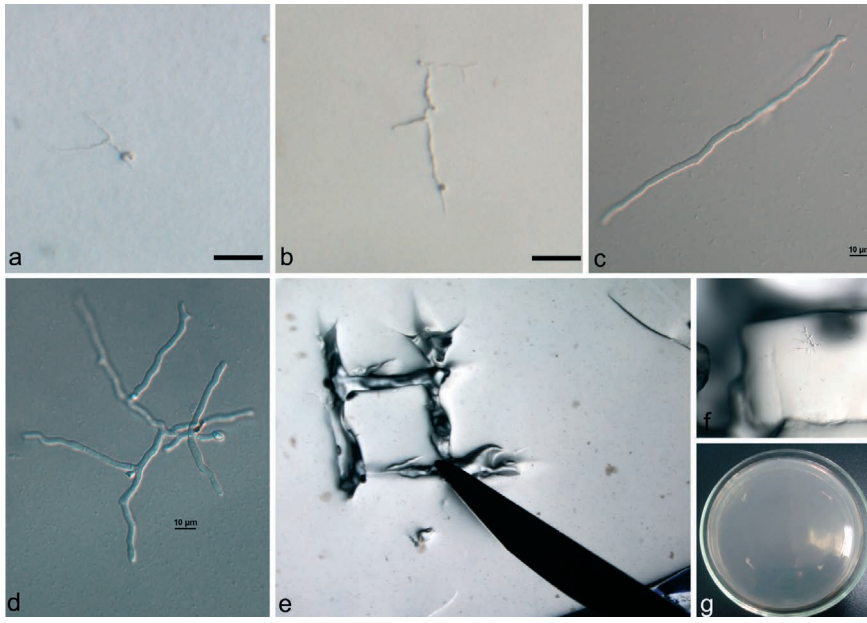

Fig. 6. Examination and transfer of the germinated spores. **a-d.** Germinating spores under dissecting microscope. **e.** Cutting media piece. **f.** Germinating spore on the media piece that has been picked up. **g.** transferred media pieces on new plate. Scale bars: a-b: 100 µm; c-d: 10 µm).

Comparison between protocols: To compare the contamination rate using the optimized protocol, a contrast experiment was conducted. Plant leaves infected by *Phyllosticta concentrica* were used. Two isolation groups (A and B) were established, each one included 20 plates. In group A, isolation was made following this optimized protocol with spore suspensions made in sterilized centrifugal tubes before spreading on plates in a laminar flow cabinet. In group B, isolation was pursued using Choi's protocol (Choi *et al.*, 1999) with spore suspensions made upon sterilized glass slides, then mixed and spread on plates beside the flame one by one. The 40 plates were sealed and cultured at ca. 25°C, and the amount of contaminated non-*Phyllosticta* colonies was recorded after 3 days.

RESULTS

After 3 days of incubation, 3 contaminated colonies were observed in group A, while 27 contaminated colonies were observed in group B (Table 1). The result showed that the optimized protocol could remarkably lower the contamination rate in the isolation. The differences between both protocols are summarized in Table 2.

Table 1. Comparision of encountered contamination between protocols

Group number	*Day 1*		*Day 2*		*Day 3*	
	Group A	*Group B*	*Group A*	*Group B*	*Group A*	*Group B*
1	–	–	–	1	–	2
2	–	–	–	2	–	2
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	–	–	–	–	–	7
7	–	–	–	–	–	1
8	–	–	–	–	2	–
9	–	–	–	–	–	1
10	–	–	–	–	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	–	–	–	–	–	–
14	–		–	–	–	–
15	–	–	–	–	–	–
16	–	2	1	3	1	3
17	–	1	–	1	–	4
18	–	1	–	1	–	1
19	–	1	–	5	–	6
20	–	–	–	–	–	–
Sum	0	5	1	13	3	27

Table 2. Comparison between the protocol of Choi et al. (1999) and our optimized protocol. Differences are marked in bold

	Choi et al.'s protocol	*Optimized protocol*
Materials and facilities	Plant material, sterilized glass needles and extra fine forceps, 70% ethanol, microscope, sterilized water, **glass container or glass slide**.	Plant material, sterilized water**,** sterilized glass needles and extra fine forceps, 70% ethanol, microscope, **centrifugal tubes**, **laminar flow cabinet**.
Preparation before isolation	Sterilization.	Sterilization. **200 µL sterilized water were added into the centrifugal tubes**.
Making spore suspension	Working beside work bench. **Preparing suspension above a glass slide**.	Working beside work bench. **Preparing suspension in a sterilized centrifugal tube**.
Spreading spore suspension	**Working beside work bench**. Suspension is transferred with sterilized micropipette.	**Working in laminar flow cabinet**. Suspension is transferred with sterilized micropipette.
Incubation	Plates are incubated and checked periodically.	Plates are incubated and checked periodically.
Transferring of germinated spores and culturing.	Working beside work bench. Finding appropriate germinated spores with dissecting microscope. Culturing on desired media.	Working beside work bench. Finding appropriate germinated spores with dissecting microscope. Culturing on desired media.
Advantages	**Lower facility request. Separate processes dealing with each sample**.	**More convenient, less experiences and skills needed. Less time consuming. High-throughput. Lower contamination rate**.

DISCUSSION

According to the results of our comparative experiment, this optimized protocol is relatively high throughput, easier and more convenient to operate and has a lower contamination rate compared to previous single spore isolation protocols. The main distinction is the use of centrifugal tubes for the treatment of spore suspensions, which allow the suspension to be mixed in batch and in a confined space, whereas the spore suspensions are then spread on agar plates in a laminar flow cabinet. Both aspects can effectively prevent contamination from non-target spores in the surrounding air. In Choi's protocol (Choi *et al.*, 1999), the operator had to prepare and mix the suspensions one by one upon a sterilized glass slide and spread the suspension onto the agar plate in an open, unprotected environment.

In addition, as these procedures have to be repeated many times when dealing with the isolation of a large number of fungi, this optimized protocol enables a more efficient and less time consuming processing of samples, as one can mix all the spore suspensions simultaneously by vortex, and then spread them on agar plates in the laminar flow cabinet.

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REFERENCES

BAAYEN R., BONANTS P., VERKLEY G., CARROLL G., VAN DER AA H., DE WEERDT M., VAN BROUWERSHAVEN I., SCHUTTE G., MACCHERONI JR W., DE BLANCO C.G., 2002 — Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). *Phytopathology* 92: 464-477.

BOOTH C., 1971 — *Methods in microbiology.* Vol. 4. Academic Press, London.

CAI L., MCKENZIE E.H.C., HYDE K.D., 2004 — New species of *Cordana* and *Spadicoides* from decaying bamboo culms in China. *Sydowia* 56: 222-228.

CHOI Y.W., HYDE K.D., HO W., 1999 — Single spore isolation of fungi. *Fungal Diversity* 3: 29-38.

GLIENKE C., PEREIRA O., STRINGARI D., FABRIS J., KAVA-CORDEIRO V. GALLI-TERASAWA L., CUNNINGTON J., SHIVAS R., GROENEWALD J., CROUS P., 2011— Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. *Persoonia* 26: 47-56.

GOH T.K., 1999 — Single-spore isolation using a hand-made glass needle. *Fungal Diversity* 2: 47-63.

HAWKSWORTH D.L., 1974 — *Mycologist's handbook: an introduction to the principles of taxonomy and nomenclature in the fungi and lichens.* Commonwealth Mycological Institute.

HU D.M., CHEN H., CAI L., BAHKALI A.H., HYDE K.D., 2012 — *Aquapeziza*: a new genus from freshwater, and its morphological and phylogenetic relationships to Pezizaceae. *Mycologia* 104: 540-546.

LIU F., HYDE K.D., CAI L., 2011 — Neotypification of *Colletotrichum coccodes*, the causal agent of potato black dot disease and tomato anthracnose. *Mycology* 2: 248-254.

SHAW B., KUO K.C., HOCH H., 1998 — Germination and appressorium development of *Phyllosticta ampelicida* pycnidiospores. *Mycologia* 90: 258-268.

WIKEE S., CAI L., PAIRIN N., MCKENZIE E.H.C., SU Y.Y., CHUKEATIROTE E., THI H.N., BAHKALI A.H., MOELEM M.A., ABDELSALAM K., HYDE K.D., 2011 — *Colletotrichum* species from Jasmine (*Jasminum sambac*). *Fungal Diversity* 46: 171-182.

WONG M.H., CROUS P.W., HENDERSON J., GROENEWALD J.Z., DRENTH A., 2012 — *Phyllosticta* species associated with freckle disease of banana. *Fungal Diversity* 56: 173-187.