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A simple method for obtaining single-spore isolates of fungi

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Abstract. A new method of single spore isolation was developed. A 0.1 μl spore suspension was placed on water agar above one of 50–100 circles marked on the bottom of a plate. After 12–24 h, the number of spores in each circle was counted. Single germinating spores in each circle were transferred separately. For all six species of fungi tested, the number of spores in each micro-drop ranged from 0 to 4. More than 50% of the micro-drops contained a single spore with an unbranched germ tube. This method made it easy to locate well separated spores for single-spore isolation, shortened the isolation time by half, and reduced the incubation period from two days to one.

Keywords: Single-spore isolation; Zoospores; Conidia.

Introduction

Establishing a large number of single-spore isolates is essential for studies of variation, mutation, and segregation in fungi. The conventional method of isolating well separated spores streaked on the agar medium under a stereoscopic microscope (Tuite, 1969) is tedious and time consuming. While studying the mating type change in *Phytophthora parasitica* Dastur, Ko (1981) developed a relatively easy method for mass isolation of single-zoospore isolates. The method consisted of streaking encysted zoospores on water agar. After incubation at 24°C for 48 h, separated visible colonies were marked and then observed under a light microscope at 100× magnification with 10× objective. Those colonies originating from single zoospores were transferred to V-8 agar plates. This method also has been used to study chemical regulation of mating type in various species of *Phytophthora* (Ko et al., 1986; Ann and Ko, 1989; Chang and Ko, 1990), to compare the enzyme activity between asexual and sexual progenies of *P. parasitica* (Ann and Ko, 1990a), to obtain genetic evidence of diploidy for this fungus (Ann and Ko, 1990b), and to document variation in growth rate and colony morphology in *P. parasitica* induced by exposure to metalaxyl (Chang and Ko, 1992). The method was also used to detect mating type changes during long-term storage of *Pythium splendens* Braun (Guo and Ko, 1991), to determine whether this fungus was diploid (Guo and Ko, 1994), and to document continuing variation in successive asexual generations of *Py. splendens* following sexual reproduction (Guo and Ko, 1995). The method developed by Ko (1981) requires time to locate the origin of each colony under the microscope to confirm that the colony originated from a single spore (Figure 1). In this report we describe a simple procedure that eliminates the need to locate the colony origins and, thereby, shortens the time needed to identity single-spore isolates.

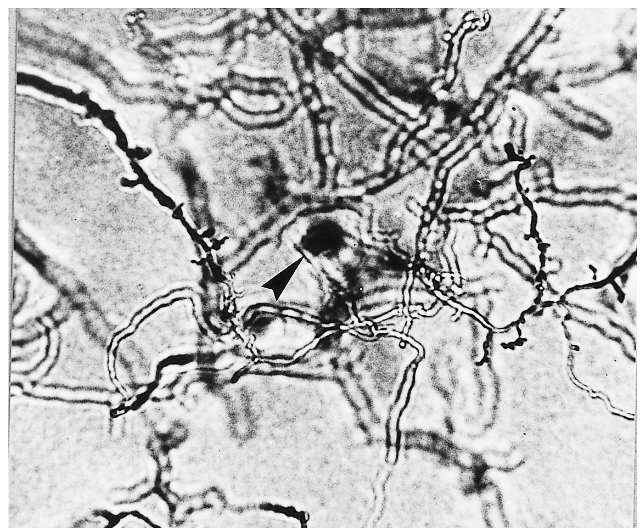

Figure 1. A small colony developed from a single zoospore (arrow) of *Phytophthora parasitica* on water agar 48 h at 24°C (320×).

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Materials and Methods

Microorganisms

Cultures of *Phytophthora megasperma* Drechsler, *P. parasitica, Pythium carolinianum* Matthews and *Py. myriotylum* Drechsler were each originated from a single zoospore. *Botryodiplodia theobromae* Pat., and *Colletotrichum gloeosporioides* (Penzig) Sacc. originated from single conidia.

Production of Spores

Phytophthora parasitica, P. megasperma, Py. carolinianum, and *Py. myriotylum* were individually grown on 5% V-8 agar (5% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) at 24°C for 3 days. Sporangia of each fungus were induced by incubating three pieces (ca. 5 × 5 × 5 mm) of the agar culture in 10 ml sterile distilled water in a plastic Petri plate (60 mm diam.) for 2 days at 24°C under cool white fluorescent light (1,000 lux). Zoospores were released from sporangia by chilling at 5°C for 15 min and were induced to encyst by agitating the zoospore suspension in a test tube for 1 min with a Vortex mixer. For production of conidia, *B. theobromae* and *C. gloeosporioides* were grown on 10% V-8 agar (10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) at 24°C for 2 weeks under light. The conidial suspension was prepared by transfering a loopful of conidial mass into sterile distilled water in a test tube.

Isolation of Single-Spore Isolates

The concentrations of the spore suspensions were adjusted to approximately 10 spores/μl with a Pipetman microliter pipet (P-20D, West Coast Scientific Inc., Oakland, California, U.S.A) (Ko et al., 1973). A marking pen was used to draw 50–100 circles (ca. 3 mm diam.) on the bottom of each water agar (2% Bacto agar) plate (9 cm diam.). A 0.1 μl drop of the spore suspension was placed on the surface of the water agar above each circle. After incubation at 24°C for 12–24 h, each circle was inspected under a microscope at 100× magnification from the bottom of the plate. Those circles containing a single germinating spore were marked and spores in those circles were individually transferred to 10% V-8 agar plate.

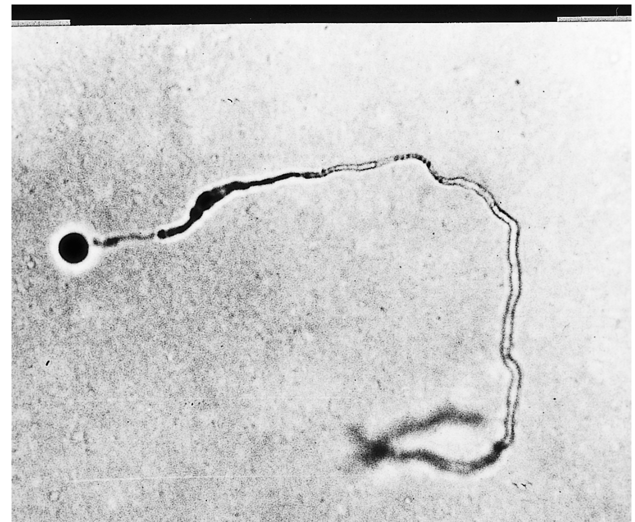

Figure 2. A zoospore of *Phytophthora parasitica* with an unbranched germ tube on water agar after 12 h at 24°C (320×).

Table 1. Ratios of spore number in mico-drops of spore suspensions adjusted to about 10 spores/μl.

Fungus	Spore type	No. of micro-drops[a] containing				
		0	1	2	3	4 (spores)
Botryodiplodia theobromae	Conidia	25	57	14	4	0
Colletotrichum gloeosporioides	Conidia	25	55	14	4	2
Phytophthora megasperma	Zoospores	31	51	12	4	2
P. parasitica	Zoospores	28	55	13	3	1
Pythium carolinianum	Zoospores	32	51	15	2	0
Py. myriotylum	Zoospores	27	52	17	3	1

[a]Each micro-drop contained 0.1 μl of spore suspension.

Table 2. Time required for isolation of 50 single-spore isolates from different fungi using the new method and the previous method[a].

Fungus	Time required (min)	
	Present method	Previous method[a]
Botryodiplodia theobromae	51	137
Colletotrichum gloeosporioides	52	130
Phytophthora megasperma	62	133
P. parasitica	72	165
Pythium carolinianum	65	131
Py. myriotylum	64	127

[a]Ko (1981).

Results and Discussion

When the spore concentration was adjusted to about 10 spores/μl, the number of spores in each micro-drop ranged from 0 to 4 (Table 1). More than 50% of the micro-drops contained a single spore, and 35% of the drops contained no spores. Approximately 14, 3, and 1% of the micro-drops contained 2, 3, and 4 spores, respectively. For example, when conidia of *B. theobromae* were tested, 57% of the drops contained a single spore, while 25, 14, and 4% of the drops contained 0, 2, and 3 spores, respectively. Within 12 to 24 h of incubation, most spores produced an unbranched germ tube making them easy to locate (Figure 2). Therefore, the time needed to identify a single-spore isolate was greatly shortened. For all six fungal species tested, the time required to obtain 50 single-spore isolates was about 60 min with the new method versus over twice that long with the old method (Table 2).

The method described in this study has the advantage of being easy to locate well separated spores for single-spore isolation. It not only shortened the isolation time by half, but also reduced the incubation time from two days to one.

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