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Abstract

Phenotypic and genotypic studies on species responsible for grey mould disease, *Botrytis cinerea* and *B. pseudocinerea*, are usually carried out on purified strains. Purification is classically achieved with the isolation of a single spore. This method entails fastidious and time consuming steps and requires a good level of experience of the manipulator. An alternative method is based on the isolation of hyphal fragments under the stereomicroscope. Both methods are widely used but their efficiency has never been formally quantified. The two methods were compared for the purification of mixes of strains with known genotypic profiles. To assess the efficiency of the purification, the resulting isolates were characterized with microsatellite markers and we verified that each represented a single genotype. Both methods had equally high efficiencies, up to 93 to 95%. The advantage of the simplified method is that it is much less time consuming than single spore isolation (2 versus 19 days) and requires less technical experience.

Keywords: spore, hypha, microsatellite, purification

1. Introduction

The necessity of using pure culture strains in studies of plant pathogenic fungi has long been advocated (Hildebrand, 1938) and is usually recognized as a standard practice before carrying out the phenotypic or genotypic characterization of an isolate. A "pure culture" of a microorganism was described by Orskov (1922) as "a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and from one only". A number of methods are available to obtain pure cultures, many of which have been described by Hildebrand (1938).

For spore producing fungi, the standard purification method is based on single spore isolation (Choi *et al*., 1999). This is the case, for example, for the plant pathogenic fungus *Botryotinia fuckeliana* (anamorph *Botrytis cinerea*). This ascomycete can produce large amounts of asexual spores on diseased plants (Jarvis, 1977; Nicot *et al*., 1996). For population studies on this fungus and on related cryptic species *B. pseudocinerea* (Walker *et al*., 2011), field samples are routinely collected from spore-bearing lesions with the help of a sterile cotton swab (Fournier & Giraud, 2008). Due to the possible presence on a same lesion of spores from isolates with different properties (Giraud *et al*., 1997; Munoz *et al*., 2002), such samples are often referred to as "micropopulations" and subjected to single spore isolation prior to characterization. The process is time consuming, fastidious and its successful accomplishment requires experience for the meticulous handling of spores (often smaller than 10µm in diameter) in sterile conditions under the microscope. This could be a limiting factor in population studies for which large numbers of strains need to be characterized. Based on methods described for non-sporulation fungi (Hildebrand, 1938), one possible easier-to-handle alternative to single spore purification is the isolation of hyphal tips from the growing margin of a colony on nutrient agar medium.

Whether based on single spore or on hyphal tip isolation, a purification technique can be considered as efficient if the resulting strains are pure from a genetic point of view. Errors with the hyphal tip isolation can result from the presence of hyphae of different strains in the piece of agar medium which is transferred. Errors with the single spore isolation can result from the inadvertent transfer of additional spores present in the close vicinity of the intended single spore. As far as we know, the efficiency of both methods has never been quantified. While in former times, mixtures of strains with nearly identical phenotypes could be difficult to detect, the advent of molecular genotyping techniques has provided a powerful tool to verify that purified strains represent a single genotype.

The present study was thus initiated to compare the efficiency of a simplified hyphal fragment isolation method to the traditional single spore isolation, using known mixtures of isolates with characterized genotypic profiles. The specific objectives were i-) to determine if the efficiency of the simplified method is equivalent to that of the single spore method, ii-) to evaluate the gain of time provided by the simplified method, iii-) to determine if there may be an isolation bias in presence of strains with different growing rates and if this bias would be similar for both methods, and iv-) to verify that both cryptic species *B. cinerea* and *B. pseudocinerea* can be isolated.

2. Material and methods

2.1. Botrytis cinerea *and* B. pseudocinerea *isolates*.

Seven single spore isolates (six of *B. cinerea* and one of *B. pseudocinerea*) were used in this study. They were selected from our collection, based on genotypic and phenotypic properties characterized in previous work (Table 1). Each isolate showed a unique allelic profile for microsatellite markers BC3 and BC6 described by Fournier *et al.* (2002). Their growth rates on malt agar, determined according to the method of Martinez *et al*. (2003), were between 15 and 17 mm day $^{-1}$ for all but one isolate (SEp 172) which had a substantially reduced growth rate of 2.5 mm day^{-1} (Table 1). The sporulation capacity of all isolates was comparable, with average amounts of 8-50 million spores produced in a Petri plate in 14 days.

2.2 Preparation of isolate mixes

In order to evaluate the efficiency of isolation methods, mixes of isolates were prepared. We sought to mimic in Petri plates the micropopulations encountered in the field on sporulating lesions. For this purpose, the seven isolates were grown for 2 weeks on potato dextrose agar (PDA, Difco) at 21°C in a climatic chamber under white fluorescent light (14-hour photoperiod; 65 µmole $m^{-2}s^{-1}$). Spore suspensions were prepared for each isolate as described earlier (Decognet *et al*., 2009) and calibrated at 10^4 spores mL^{-1}. Aliquots from each suspension were spread on fresh PDA and their germination rate was assessed 24 hours after inoculation.

Mixed cultures of the different isolates were prepared by spreading 100 µL aliquots of each of two or three spore suspensions of different isolates on fresh PDA Petri plates. In total 23 mixes were prepared: 17 combinations of two isolates and 6 combinations of three isolates (Table 2). All this work was carried out in sterile conditions under a laminar flow hood to avoid contaminations. The plates were incubated at 21°C for 2 weeks under cool fluorescent light as indicated above. From each mix, spores were then collected with sterile cotton swabs and stored in micro-tubes at -20°C until used for purification.

2.3 Isolation of isolate mixes

Each mix was purified using two methods: single-spore isolation and a simplified hyphal fragment isolation method. The first step for both types of methods consisted in rubbing a spore-bearing cotton swab on the center of a Petri plate filled with nutrient agar (Table 3). This step was intended to mimic the first isolation carried out when samples are brought back from the field. While PDA was used for the single spore isolation, malt agar was used for the hyphal fragment isolation because the growth of the fungus on this latter medium is sparse, allowing the observation of well separated individual hyphae at the margin of the colony.

*Single spore isolation method***.** Two days after inoculation with the cotton swab, a mycelial plug was removed from the growing margin of the colony and transferred to a fresh PDA plate. After two weeks of incubation, sporulating colonies were gently brushed with a wet sterile loop and the collected spores were put in 1mL of sterile water and vortexed. A loopful of this suspension was then spread on a thin layer of sterile water agar coated over a microscope slide in order to separate the spores. Twenty-four hours later, a small block of medium (ca 1x1x1 mm) carrying a single germinated spore was collected in sterile conditions under the microscope (400 x) and transferred to a fresh PDA plate. Two days later, a mycelial plug was excised from the periphery of the young colony initiated by the single spore and transferred to a fresh PDA plate. The purified strain was then put in collection.

Hyphal fragment isolation method. Two days after inoculation with the cotton swab, the surface of the MA plate was examined under the binocular (100x) to identify a well separated hypha. A small block of medium (ca 2x2x1 mm) carrying a fragment of this hypha was excised in sterile conditions and transferred to a fresh PDA plate. The purified strain was then put in collection.

For both methods, two replications of the isolation were carried out with each isolate mix.

2.4 Isolate genotyping

Each purified strain was grown for 14 days on PDA as described above (paragraph 2.2). Fungal material (mycelium and spores) were collected, lyophilised and stored at -20°C until used. DNA extractions were done on 15 mg aliquots of lyophilized material, using a QIAGEN DNeasy 96 Plant Kit (QIAGEN, Venlo, the Netherlands) and following the manufacturer's instructions for "purification of total DNA from frozen lyophilized plant tissue". The DNA was then amplified using the multiplex PCR kit (QIAGEN) and following the manufacturer's instructions for amplification of microsatellite loci. Four reference isolates with known genotypic profiles were included as controls. Microsatellite markers BC3 and BC6 were amplified at 50°C. Forward primers were labelled with fluorescent dye HEX for BC6 and TAMRA for BC3. Allele sizes were determined using a 48-capillary sequencer (ABI 3700, Applied Biosystems). The analyses of allele sizes were carried out with GeneMapper software (Applied Biosystems, Foster City, USA).

2.5 Evaluation of isolation efficiency

As *B. cinerea* and *B. pseudocinerea* are haploid microorganisms successful isolations from the different mixes should each reveal only one allele for each of the microsatellite markers. The efficiency of the two isolation methods was thus assessed independently for each marker as the proportion of isolated strains showing a single allele.

3. Results and Discussion

3.1 Efficiency of the isolation methods

While the majority of purified strains showed a unique amplified allele with either the BC3 or the BC6 marker, multiple alleles were observed in some cases, suggesting the presence of DNA from several strains (Table 2). The two methods showed identical isolation efficiency, estimated at 93.4% when assessed with microsatellite BC6 and 95.0% with BC3. An exact test of Fisher confirmed that the proportion of purified strains showing multiple alleles from the 23 isolate mixes was independent of the isolation method, either when assessed with BC6 or BC3 (with P values $P>0.71$ and $P>0.99$, respectively).

Two hypotheses can be proposed to explain the presence of multiple alleles. It may be due to the failure of the isolation method. The observation of multiple alleles could also result from the phenomenon of heterokaryosis suspected to occur in field isolates of *B. cinerea* (Beever and Weeds, 2004) as this fungus is known to have several nuclei in each spore and mycelial cell (Shirane *et al*., 1988).

3.2 Compared practicality of the isolation methods

In total, 19 days were necessary for single spore isolation, while hyphal fragment isolation required only 2 days. Thus, purified strains can be obtained much more rapidly with hyphal fragment isolation. The simplified method also provides substantial time saving as it requires fewer steps (2 vs. 5) and fewer material items (for example 2 vs. 4 nutrient agar Petri plates; no need for sterile agar-covered glass slides), hence less preparation time. Furthermore, the excision of a hyphal fragment under the binocular is simpler and requires less skill than collecting a single spore under the microscope. nutrient agar Petri plates; no need for sterile agar-covered glass slides), hence less preparation time. Furthermore, the excision of a hyphal fragment under the binocular is simpler and requires less skill than collecting a single spore under the microscope. However, dense, compact mycelia can make the hyphal fragment method difficult; this can be mitigated with *Botrytis* spp. by using a medium on which the mycelium grows more sparsely.

3.3 Isolation and growth rate

In seven of the isolate mixes, slow growing strain SEp 172 was introduced with fast-growing strains, in order to determine if the choice of an isolation method may have an impact on which strain is isolated in the end. With both purification methods, the alleles of the fast growing strain, but not those of strain SEp 172, were always detected in the purified culture. Thus, both techniques tended to exclude the slow growing strain. As germination rates were between 98 and 100% for all strains used in this study, we assume that they all germinated and developed in the first step of the isolate mix preparation. However, it was not possible to verify if similar numbers of spores of the slow and fast growing strains were present on the cotton swabs obtained from the mix plates.

3.4 Isolation of B. cinerea *versus* B. pseudocinerea

The *B. pseudocinerea* strain M330 was introduced with some *B. cinerea* strains in eight mixes (Table 2). The frequency of *B. pseudocinerea* isolation versus *B. cinerea* was identical for both isolation methods. For the results with both microsatellite loci, the two-tailed P value of the exact Fisher's tests were nearly 1, thus the repartition of the two species obtained after isolation was independent of the technique used.

4. Conclusion

The present study showed that a method based on the isolation of hyphal fragments and a method based on the isolation of single spore are suitable for strain isolation of *B. cinerea* and *B. pseudocinerea*. Their efficiency was identical and high (between 93 and 95%). The simplified method has the advantage of being less time consuming and less dependent on skilled experience than the classical one. As a first step in a population study, this method will allow purifying many more isolates in less time while being efficient. Conceivably, it could be performed equally well for other fungal species with characteristics similar to those of *B. cinerea* and *B. pseudocinerea* (ability to grow on agar media, same size of hyphae for example). Moreover, it could be particularly useful for fungal species that do not sporulate well on synthetic culture medium.

5. References

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Table 1: Growth rate, sporulation capacity and allele sizes for microsatellite alleles (BC6 and BC3) of strains of *Botrytis* spp. grown in mixtures with *B. cinerea*.

Strain names	Growth rate (mm. day $^{-1}$ ± standard deviation)	Sporulation (x10^6 spores per plate [a] ± standard deviation)	BC6 allele[b] (size in bp)	BC3 allele[b] (size in bp)
Bc1	17.2 ± 0.0	17 ± 3	114	219
SEp 169	17.2 ± 0.0	26 ± 2	150	215
SEp 189	16.2 ± 2.1	50 ± 12	126	215
SEp 184	16.2 ± 1.0	17 ± 3	76	235
SEp 29	15.5 ± 2.5	22 ± 1	118	215
M330[c]	15.0 ± 0.8	8 ± 1	84	203
SEp 172	2.5 ± 1.0	17 ± 1	118	225

a: Petri plates were 8.5 cm in diameter.
b: alleles determined with sequencer ABI 3730 and GeneMapper software (Applied Biosystems)
c: strains were *Botrytis cinerea*, except M330, which was *B. pseudocinerea*.

Table 2: Compared efficiency of the single spore isolation method (SSM) and the hyphal fragment isolation method (HFM), based on the detection of BC6 or BC3 microsatellite alleles, for detecting *Botrytis cinerea* in mixture with two or three strains of *Botrytis* spp.

Mix	Composition of the strain mixes							Number of single-allele detections in the isolation results[b]			
	Strains of *Botrytis* sp.[a]							SSM		HFM	
	Bc1	M330	SEp 189	SEp 169	SEp 172	SEp 29	SEp 184	BC6	BC3	BC6	BC3
M1	x	x						1	2	2	2
M2	x		x					2	2	2	2
M3	x			x				2	2	2	2
M4	x				x			2	2	2	1[c]
M5	x					x		2	2	2	2
M6	x						x	2	2	2	2
M7		x	x					2	2	2	2
M8		x		x				2	2	2	2
M9		x			x			2	2	2	2
M10		x				x		2	2	2	2
M11		x					x	1	1	1	0[c]
M12			x	x				2	2	2	2
M13			x		x			2	2	1	2
M14			x			x		2	2	2	2
M15			x				x	2	2	2	2
M16				x	x			1	1	1	1[c]
M17				x		x		2	2	2	2
M18	x	x	x					2	2	2	2
M19	x	x		x				2	2	1[c]	1[c]
M20			x	x	x			2	2	1[c]	1[c]
M21				x	x	x		2	2	1[c]	1[c]
M22				x		x	x	2	2	2	2
M23	x				x		x	2	2	2	2
Overall isolation efficiency[d]								93.4%	95.6%	93.4%	95.0%

[a] : Strains were *Botrytis cinerea*, except M330 which was *B. pseudocinerea*.

[b]: Four replicate isolations were performed from each mix (two with each isolation method). Detecting a single allele with microsatellite marker BC3 or BC6 was taken as indicative that a single strain had successfully been isolated from the mix; conversely, detecting more than one allele indicated that the isolation procedure had failed.

[c] : allele not readable in one well (problem of marker size).

[d] : Isolation efficiency was calculated as percentage of attempts resulting in pure cultures based on detection of only the single microsatellite marker (BC6 or BC3). The Fisher exact test showed no significant difference in the isolation efficiency between the SSM and HFM methods for detecting strains BC6 and BC3 ($P = 0.71$ and $P > 0.99$, respectively).