RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.086

ScienceAsia 48 (2022): **764–768**

A novel, rapid and simple method for obtaining single-spore isolation of strongly parasitic fungi from diseased cherry leaves

Xiuming Ma, Jun Miao, Junfeng Wang, Wei Han*, Yang Sun*

Institute of Vegetables and Flowers, Shandong Academy of Agricultural Sciences, Shandong Provincial Key Laboratory of Vegetable Biology, Shandong Branch of National Center for Vegetable Improvement, Jinan 250100 China

*Corresponding authors, e-mail: sdscshw203@126.com, sunyang_729@163.com

Received 22 Jan 2021, Accepted 27 Feb 2022
Available online 10 Aug 2022

ABSTRACT: Spore isolation is an essential experimental approach in the fields of mycology and plant pathology, enabling researchers to isolate fungi from diseased plant samples for subsequent identification. Routine single-spore isolation strategies, however, are time-consuming and highly susceptible to contamination with other microorganisms. Herein, we developed a rapid approach to isolate individual spores of the fungal pathogen *Passalora circumscissa* from diseased cherry leaves in an orchard with a low risk for contamination. Diseased cherry leaves with individual lesions were first selected from the orchard, sterilized with 75% ethanol, and rinsed under deionized water. An acquisition tool was then used to isolate a single spore that was absorbed from the disease spot, and the spore was transferred onto the surface of water agar plate. Individual germinating spores were then excised from these agar plates using a cutting tool under a 10 × objective magnification microscope. Lastly, the germinating spores were transferred from the water agar plates onto potato agar media for further growth and preservation. Based upon our findings, an experienced technician can utilize this strategy to isolate individual spores from over 100 independent diseased plant tissue samples in a single day with minimal risk of contamination. This strategy should also be well-suited for the isolation of individual fungal spores associated with other diseased sample types.

KEYWORDS: cherry, *Passalora circumscissa*, single spore isolation

INTRODUCTION

Cercosporoid make up the largest ascomycetes genera composing of approximately 2000 species that can cause leaf spot, fruit spot, and blight diseases in many different plants worldwide [1–5]. *Cercosporoid hyphomycetes* classification is primarily based on factors including host specificity, morphological characteristics, and molecular phenotyping. Morphological changes in these fungi due to variable culture conditions, however, can lead to their misidentification. Molecular approaches are, therefore, often used to overcome these morphology-based limitations to fungal taxonomic classification and various new species or re-identification has been reported [6, 7]. The analysis of conserved rDNA sequences represents an efficient and reliable approach to systematically classify and identify fungi via molecular approaches. However, conducting these molecular analyses is dependent upon obtaining a pure pathogen culture from which genomic DNA can be isolated.

The two primary strategies that have been employed to obtain pure fungal cultures are surface sterilization and single spore isolation [8]. Surface sterilization strategies, however, are limited in their ability to yield pure cultures of obligate parasitic pathogens as these pathogens must obtain nutrients from live plant tissues and often grow slower than saprophytes on artificial media. In addition, pathogens within plant tissues are often mixed with other endophytic bacteria, saprophytes, and miscellaneous bacteria that can contaminate cultures of isolated pathogens, leading to erroneous molecular phenotyping. As such, single spore separation strategies are more commonly utilized to separate and analyze these pathogens of interest.

Single-spore isolation approaches are commonly employed in studies designed to characterize, purify, or preserve fungal samples. Several different single spore isolation methods have been described to date. Matsushima [9] utilized a micromanipulator to isolate and arrange individual fungal spores for photographic imaging in his mycological lectorum, while Constantinescu [10] developed an improved instrument that was attached to a microscope objective and facilitated rapid single-spore isolation for analyses of various fungi. Contrarily, another study [11] utilized a capillary pipette to isolate individual target spores from a spore suspension and transferred them onto a glass slide. Besides, another research [12] detailed a micromanipulation strategy, wherein bacteria or fungi were transferred from agar plates using microcapillary tubes. Previous studies [13–15] also generated practical approaches to isolate individual spores with glass needles, and these strategies have subsequently been adopted by a number of other studies [16–19].

These approaches, however, are dependent upon the availability of specialized equipment and well-trained technicians. Micromanipulators are very costly and may not be accessible to some laboratories. In

addition, significant skill is necessary to accurately aspirate spores into a fine capillary tube and to subsequently break these tubes into short fragments containing individual spores.

Given the limitations of these extant approaches, the present study sought to develop an efficient and straightforward approach to single-spore isolation that can be readily conducted by researchers without uses of specialized equipment. The approach described herein does not necessitate a costly micromanipulator and requires less skill than do capillary tube-based methods or approaches that require specialized instrument production.

In the present study, we utilized *Passalora circumscissa*, (modern classification from *Cercospora circumscissa* or *hyphomycetes*), as a model and demonstrated that our strategy enables efficient single-spore isolation in an inexpensive, user-friendly, and reliable manner. The approach only needs the use of basic equipment, meaning that even laboratories with limited funding can execute the protocols described herein.

MATERIALS AND METHODS

Materials

Basic materials for this single-spore isolation approach included glass slides, a general optical microscope, a dissecting needle, 100 µl pipette tips, 2.5 µl pipette tips, an alcohol burner, a clean bench space, micropipettes, flat tweezers, 3% water agar, and potato dextrose agar (Fig. S1). All agars were prepared via autoclaving at 121 °C for 20 min, then antibiotics were added when the agars had cooled to 50 °C.

Spore visualization and isolation were conducted with a general optical microscope. Spores on the water agar slice were incubated in plastic containers for germination. The spore acquisition tool used herein was constructed using sterilized 2.5 µl pipettes, a dissecting needle, spirit lamps, and flattened tweezers.

Methods

Acquisition tool preparation

The spore acquisition tools consisted of a dissecting needle and two types of modified pipettes, including an attachment tool and a cutting tool (Fig. S2).

The attachment tool was prepared by briefly heating a 2.5 µl pipette tip over an alcohol lamp until it melted and became rounded. Then, the tip was removed from the flame and cooled. The round and smooth pipette tips allowed spores from diseased spot to adhere to the spheroid surface prior to transferring them onto water agar slices without the need to scratch the leaf lesions.

The cutting tool was prepared by melting the front end of a 2.5 µl pipette tip over an alcohol until it melted and became rounded. Then, the tip was removed from the flame. Tweezers were used to pull the melted head of the pipette tip, followed by the use of scissors to generate a pointed, flattened spatula.

Fig. 1 Diseased spots were crushed and repeatedly smeared, and spores were absorbed using the attachment tool.

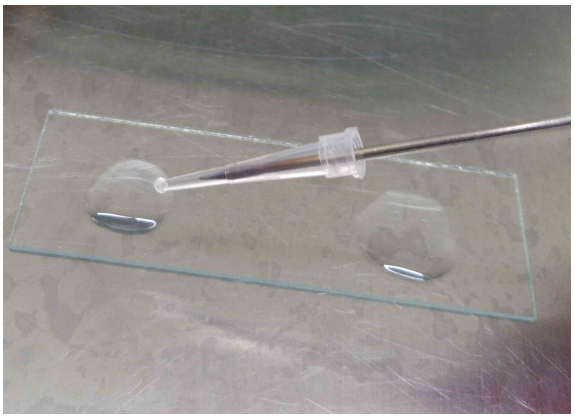

Fig. 2 Spores on the spheroid surface of the attachment tool were transferred to water agar slices.

Water agar slices (10 mm in diameter, 2 mm thick) were prepared from 100 µl of 3% water agar that was absorbed and transferred onto glass slides via micropipettor.

Procedure

Fresh diseased leaves collected from an orchard were first sterilized using 75% ethanol and, then, washed thrice with sterilized deionized water containing 2% lactic acid. The attachment tool was mounted onto the tip of a dissecting needle and briefly dipped into deionized water containing 2% lactic acid. The fruiting bodies located on the diseased leaf were then gently crushed and smeared repeatedly using the attachment tool, and the tool was used to streak repeatedly across water agar slices to ensure spore adherence to the surface of the agar slices (Fig. 1 and Fig. 2).

These water agar slices on glass slides were then examined under a general optical microscope in order

Fig. 3 One spore on a water agar slice as visualized via an optical microscope (10 × objective). Bar scale = 20 μm.

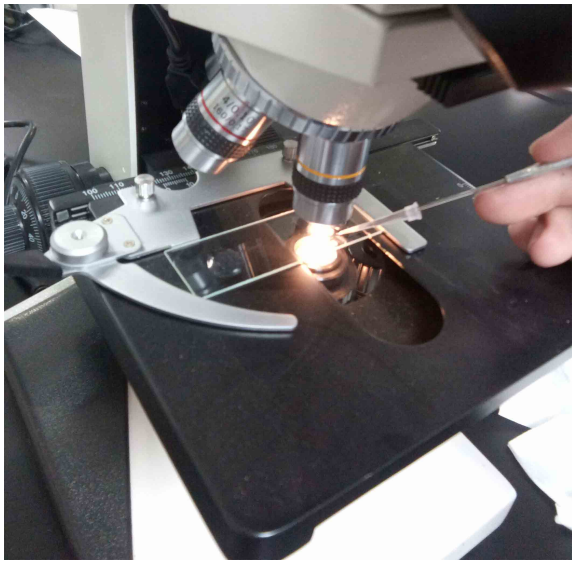

Fig. 4 A glass slide with a water agar slice on the stage was adjusted such that only one spore was located in the visual field, after which the agar was cut with the cutting tool.

to assess the number of spores in the visual field using a 10 × objective to ensure that there were just 2–3 target spores within each water agar slice (Fig. 3).

Glass slides containing these sections were then transferred into a plastic box, covered with parafilm, and incubated at 25 °C under a fluorescent lamp. Spores were assessed within 12 h and then evaluated every 24 h thereafter to assess germination. For the assessments, slides were placed on the microscope stage, and germinating spores at the agar surface were identified. When germinating spores were present, the cutting tool was used to isolate a small piece of agar containing the target spore (Fig. 4).

The cut section of media containing the target spore was then transferred to a 90 mm plate using the

Fig. 5 Infected cherry leaf 3 weeks after inoculation with *Passalora circumscissa* for demonstration of pathogenicity.

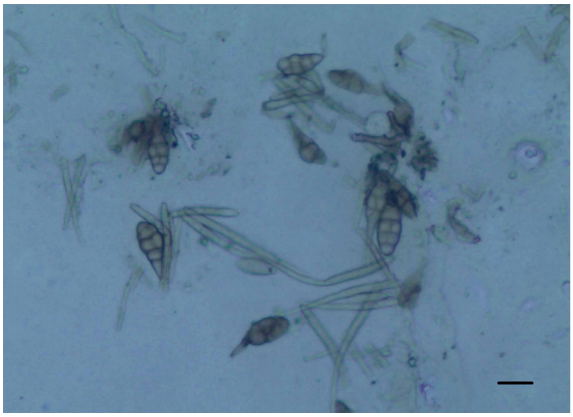

Fig. 6 *Passalora circumscissa* spore suspension containing *Alternaria* sp. spores and debris. Bar scale = 20 μm.

sterilized cutting tool. Individual spores were cultured on potato agar medium (Fig. 5).

All these protocols were conducted under sterile conditions within a laminar flow hood to avoid the potential for contamination.

RESULTS AND DISCUSSION

Single-spore isolation is an approach that is commonly used to study plant diseases by purifying pathogens and subjecting them to morphological or molecular analyses, in addition to characterizing their biochemical or genetic characteristics [20–22].

In prior studies, surface sterilization of diseased tissue was typically done with alcohol or sodium hypochlorite prior to moist cultivation, and diseased tissues were then generally re-cultured for 2–3 days to regenerate aerial fungal spores and washed with sterile water to form a spore suspension.

Alternatively, fine tweezers or scraping tools can be used to directly isolate fruiting bodies to prepare a spore suspension [14, 23]. These routing approaches,

however, are very time-consuming and highly susceptible to contamination by microorganisms other than the pathogens of interest (Fig. 6). Novel approaches to directly isolate spores from diseased tissues can reduce sample collection time and decrease the risk of off-target contamination. The spherical surface of the attachment tool developed in the present study can increase the contact area between this tool and the fruiting bodies, thereby decreasing the odds of unintentionally collecting debris or impurities. This approach relies upon collecting spores by exploiting water tension between the diseased spot and the attachment tool.

Dissecting needles, capillaries, glass needles, and other special instruments are commonly used to collect spores. However, each of these tools is associated with certain disadvantages. For example, dissecting needles are soft, while glass needles are very fragile, necessitating special care during handling. These limitations can affect the success of single spore isolation or can decrease the overall efficiency of such approaches. The handmade cutting tool used in the present study, in contrast, can readily manipulate and modify pieces of agar as necessary.

The water agar slices used in our approach provided optimal transparency when used in combination with glass slides, enabling investigators to cut the agar and adjust slides freely on the microscope stage. When 2–3 spores were transferred to each of these slides, the low spore density and absence of leaf debris, together with the sufficient operative space created by this approach, made it relatively easy to transfer pathogenic spores of interest onto PDA (Table S1).

In summary, we developed a novel approach to the isolation of single fungal spores by directly collecting spores from the surface of diseased leaves with a handmade attachment tool constructed from a 2.5 μl pipette tip. Microscopic guidance was then used to further isolate and subculture individual spores. All the necessary instrumentation for this approach was commonly available in the laboratory of phytopathology, and no special instruments (such as a high magnification dissecting microscope, a micromanipulator, or an electron microscope) were required.

CONCLUSION

In conclusion, the single-spore isolation procedure described in this study allows for the rapid and reliable isolation of individual fungal spores from diseased samples under microscopic visualization using only handmade tools. Our technique is relatively more convenient and easier to implement than previously described conventional spore isolation strategies, meaning that this approach has significant promise for future research applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.086.

Acknowledgements: This work was supported by Shandong Provincial Natural Science Foundation (ZR2014CP017) and Shandong Agricultural improved variety project (2016LCGC035).

REFERENCES

1. Crous PW, Braun U, Hunter GC, Wingfield MJ, Verkley GJM, Shin HD, Nakashima C, Groenewald JZ (2015) Phylogenetic lineages in *Pseudocercospora*. *Stud Mycol* **75**, 37–114.
2. Groenewald JZ, Nakasima C, Nushukawa J, Shin HD, Park JH, Jama AN, Groenewald M, Braun U, et al (2013) Species concepts in *Cercospora*: spotting the weeds among the roses. *Stud Mycol* **75**, 115–170.
3. Amaradasa BS, Madrid H, Groenewald JZ, Crous PW, Amundsen K (2017) *Porocercospora seminalis* gen. et comb. nov. the causal organism of buffalo grass false smut. *Mycologia* **106**, 77–85.
4. Braun U, Crous PW, Nakashima C (2014) Cercosporoid fungi (*Mycosphaerellaceae*) 2. Species on monocots (*Acoraceae* to *Xyridaceae*, excluding *Poaceae*). *IMA Fungus* **5**, 203–390.
5. Bakhshi M, Arzanlou M, Babai-ahari A, Groenewald JZ, Crous PW (2015) Is morphology in Cercospora a reliable reflection of generic affinity? *Phytotaxa* **213**, 22–34.
6. Amc T, Jeewon R, Hyde KD (2009) A re-evaluation of the evolutionary relationships within the *Xylariaceae* based on ribosomal and protein-coding gene sequences. *Fungal Divers* **34**, 127–155.
7. Chang JY, Liu SS, Shi J, Guo N, Zhang HJ, Cheng J (2020) A new *Curvularia lunata* variety discovered in Huanghuaihai Region in China. *J Integr Agric* **19**, 551–560.
8. Hawksworth DL (1974) *Mycologist's Handbook: An Introduction to the Principles of Taxonomy and Nomenclature in the Fungi and Lichens*, Commonwealth Mycological Institute.
9. Matsushima T (1975) *Icones Microfungorum a Matsuskima Lectorum*, Matsushima, Kobe.
10. Constantinescu O (1988) An instrument and procedure for single-spore isolation. *Trans Brit Mycol Soc* **91**, 700–702.
11. Wang CH, Wen HK (1997) A simple method for obtaining single spore isolate of fungi. *Bot Rev* **38**, 41–44.
12. Luttermann K, Diessel E, Weidauer M (1998) *Device for Separation of Microobjects*, Patent WO 98/03628.
13. Choi Y, Hyde K, Ho W (1999) Single spore isolation of fungi. *Fungal Divers* **3**, 29–38.
14. Goh TK (1999) Single-spore isolation using a hand-made glass needle. *Fungal Divers* **2**, 47–63.
15. Vieira W, Michereff S, Morais M, Hyde K, Camara M (2014) Endophytic species of *Colletotrichum* associated with mango in northeastern Brazil. *Fungal Divers* **67**, 181–202.
16. Wikee S, Cai L, Pairin N, McKenzie EHC, Su YY, Chukeatirote E, Thi HN, Bahkali AH, et al (2011) *Colletotrichum* species from Jasmine (*Jasminum sambac*). *Fungal Divers* **46**, 171–182.

17. Han YC, Zeng XG, Xiang FY, Zhang QH, Guo C, Chen FY, Gu YC (2018) Carbendazim sensitivity in populations of *Colletotrichum gloeosporioides* complex infecting strawberry and yams in Hubei Province of China. *J Integr Agric* **17**, 1391–1400.
18. Liu F, Hyde KD, Cai L (2011) Neotypification of *Colletotrichum coccodes*, the causal agent of potato black dot disease and tomato anthracnose. *Mycology* **2**, 248–254.
19. Hu DM, Chen H, Cai L, Bahkali AH, Hyde KD (2012) Aquapeziza: a new genus from freshwater and its morphological and phylogenetic relationships to *Pezizaceae*. *Mycologia* **104**, 540–546.
20. Mishra A, Ratnam W, Bhuiyan MAR, Ponaya A, Jena KK (2015) Single spore isolation and morphological characterization of local Malaysian isolates of rice blast fungus *Magnoporthe grisea*. *AIP Conf Proc* **1678**, 020019.
21. Leyronas C, Duffaud M, Nicot PC (2012) Compared efficiency of the isolation methods for *Botrytis cinerea*. *Mycology* **3**, 221–225.
22. Nasir B, Ijaz S, Awan FS, Haq IU (2021) Genome-wide probing of NBS-LRR encoding genes in red clover (*Trifolium pratense* L) for the identification of resistance gene analogs in *Trifolium alexandrinum* L. *ScienceAsia* **47**, 425–433.
23. Zhang K, Su YY, Cai L (2013) An optimized protocol of single spore isolation for fungi. *Cryptogam Mycol* **34**, 349–356.

Appendix A. Supplementary data

Table S1 Isolation rates of different isolation methods after 7 days of cultivation on PDA.

Method	Amount of isolation	Target colony	Contaminated colony	Isolation rate (%)
Surface sterilization	40	0	36	0
Fungi suspension dilution	40	5	28	12.5
Improved single spore isolation	40	34	1	85

Fig. S1 Materials and apparatus used in the isolation procedure.

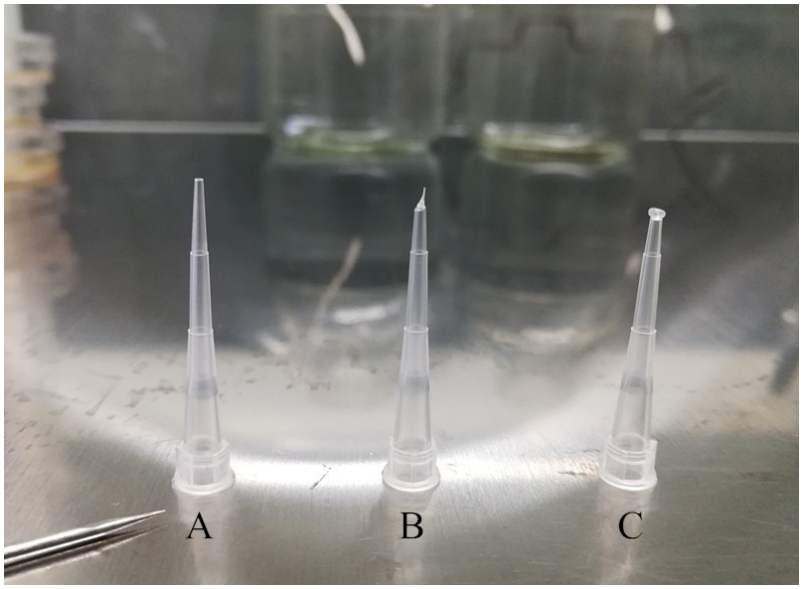

Fig. S2 Improved spore acquisition tools: A, normal pipette; B, cutting tool; C, attachment tool.