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SHORT NOTE

New method for arbuscular mycorrhizal fungus spore separation using a microfluidic device based on manual temporary flow diversion

K. Srisom[1] · P. Tittabutr[2] · N. Teaumroong[2] · Y. Lapwong[3] · R. Phatthanakun[4] · S. Sirivisoot[1] · P. Kuntanawat[2]

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Abstract

Arbuscular mycorrhizal fungi are beneficial components often included in biofertilizers. Studies of the biology and utilization of these fungi are key to their successful use in the biofertilizer industry. The acquisition of isolated spores is a required step in these studies; however, spore quality control and spore separation are bottlenecks. Filtered and centrifuged spores have to be hand-picked under a microscope. The conventional procedure is skill-demanding, labor-intensive, and time-consuming. Here, we developed a microfluidic device to aid manual separation of spores from a filtered and centrifuged suspension. The device is a single spore streamer equipped with a manual temporary flow diversion (MTFD) mechanism to select single spores. Users can press a switch to generate MTFD when the spore arrives at the selection site. The targeted spore flows in a stream to the collection chamber via temporary cross flow. Using the device, spore purity, the percentage of spore numbers against the total number of particles counted in the collecting chamber reached 96.62% (median, $n = 10$) which is greater than the spore purity obtained from the conventional method (88.89% (median, $n = 10$)).

Keywords Arbuscular Mycorrhiza · Spore separation · Microfluidic device · Manual temporary flow diversion mechanism

Introduction

Arbuscular mycorrhizal fungi (AMF) live symbiotically with plant roots and are known to benefit plant hosts in multiple ways (Amaya-Carpio et al. 2009; Nouri et al. 2014). (Göhre and Paszkowski 2006; Akhtar et al. 2011; Nouri et al. 2014; Tamayo et al. 2014; Gehring et al. 2017). These capabilities of AMF are exploited in crop production. The spores of AMF are added as an active ingredient in biofertilizer. Biofertilizer is a major industry with great potential which had a market size in 2017 of around USD 202.1 million (Gosling et al. 2006; El Kinany et al. 2018).

The success of the AMF-based biofertilizer industry very much relies on fundamental knowledge of AMF. Ranging from genetic diversity (Sanders et al. 1995; Manoharachary et al. 2002; Sasvári et al. 2012), ecology (Varga et al. 2014), physiology (Hanlon and Coenen 2011), and media preparation for inoculant production (Douds et al. 2010; Ahmad et al. 2011), studies have helped improve AMF utilization. It is arguable that spore separation from unwanted particles (i.e., particulate culture substrates, debris of plants, and soil organisms) is a key step underlying the progress of AMF studies. Without separated spores, studies cannot be systemized and controlled easily.

Presently, the standard method for spore separation is a combination of wet sieving, centrifugation, and manual spore picking (Gerdemann and Nicolson 1963; Simon et al. 2014). During these methods, however, other particles of similar density are likely to float to the surface together with the target

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✉ P. Kuntanawat
panwong.kun@g.sut.ac.th

[1] Nanoscience and Nanotechnology Graduate Program, Faculty of Science, King Mongkut's University of Technology Thonburi, 126 Pracha Uthit Rd, Bang Mod, Thung Khru, Bangkok 10140, Thailand

[2] Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand

[3] School of Life Science, University of Technology Sydney, PO Box 123, Broadway, Sydney, NSW 2007, Australia

[4] Synchrotron Light Research Institute, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand

spores. Therefore, separation of spores using sieve filters of fine diameters is required in order to eliminate contaminating particles as much as possible. Although the first two steps remove a notable amount of unwanted particles, the resulting suspension still may be considerably contaminated (about 50% based on our separation experiment using sand and vermiculite as the substrate). To achieve high purity, spore picking is required; however, it is time-consuming and skill-dependent. It is a bottleneck in AMF research.

Currently, there are only a few existing particle separation technologies potentially applicable for AMF spore separation. Flow cytometry is one of the most established methods for rapid analysis of multiple characteristics of single cells, such as physical and chemical characteristics (Bushnell and Trotter 2014). A wide variety of biological samples can be separated and sorted using this technique, such as blood cells (Roussel et al. 2010), planktonic cells (Becker et al. 2002), bacteria (Ehlers et al. 1989), and cancer cells (O'Brien et al. 2007). Samples used with flow cytometers, however, have to be well-characterized in terms of size, shape, and internal complexity to set up the sorting mode and criteria. Unfortunately, this is not the case for the separation of (total/viable) spores of a single or multiple fungus species from a pot culture. This is even worse if the spore containing substrate is a field soil. Although the spores' shape, size, and color are relatively consistent, those of contaminating particles (i.e., soil grains, culture materials, plant roots, and other soil organisms) are immensely diverse. Processing uncharacterized samples also may cause machine clogging (Yoshimoto et al. 2013). In addition, samples have to be surface-modified (i.e., fluorescent tagging), which makes separation costly and complicated (Simon et al. 2014). It also has been argued that cells put into the machine would encounter mechanical stresses and high voltages causing cell damage. Users require technical skills and knowledge to properly operate the machine, in addition to being cost intensive (Yang et al. 2006).

Emergence of microfluidic technology has led to efficient handling and manipulation of a small amount of fluid and particles within it (Wyatt Shields et al. 2015). The technology has been applied in many biological applications including cell/particle separation. Microfluidic separation techniques roughly can be divided into passive and active. The active techniques are those that employ external energy such as electric fields (Kuczenski et al. 2011; Song et al. 2015; Sadeghian et al. 2017), magnetic fields (Pamme and Wilhelm 2006; Liang and Xuan 2012; Shen et al. 2012; Distefano 2015; Huang et al. 2017), or acoustic fields (Petersson et al. 2004; Laurell et al. 2007; Amigoni et al. 2010; Shields et al. 2014). Passive techniques, on the other hand, rely on geometry and hydrodynamic forces that depend on geometric microstructure: for example, microfiltration (Yamada and Seki 2005; Ji et al. 2008; Dijkshoorn et al. 2017), pinched-flow fractionation deterministic lateral displacement (Holm et al. 2011; Jing et al. 2015), and inertial focusing (Zhang et al. 2017). However, the technologies were mainly developed to be used with samples containing two types of well-characterized particles (i.e., separation of parasites from human blood, separation of human mesenchymal stem cells from osteoblasts, or separation of white blood cells from whole blood). Although the technologies have proven to be efficient and automatic, each developed technique is usually specific to the well-known particles. For these reasons, previously developed technologies are unsuitable for use with AMF spores. The development of techniques for AMF spore separation remains a technological challenge; however, the development of technology that augments a manual protocol seems to be another possible strategy.

Here, we propose a microfluidic chip integrated with a microelectronic device for separating AM spores from a sieved and centrifuged suspension as an alternative to conventional hand-picking. Instead of a fully automated system, the technology was designed to aid manual separation. Although it needs human intervention, it makes the operation much simpler requiring less effort than hand-picking. The device is semi-automatic; it automatically lines up spores in a single row that flows into a long channel. The device allows the user to examine spores one-by-one under a microscope and to collect selected spores in a collection chamber, by pressing a switch connected to a microvalve to generate a manual temporary flow diversion (MTFD). To validate the device, the feasibility and efficiency of the separation were compared with those of the traditional method. *Rhizophagus irregularis* spores were used in the validation study.

Materials and methods

Mycorrhizal fungus species and culture

Spore pot cultures (a mixture of spores in sand and vermiculite) of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* were provided by the Applied Soil Microbiology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. The initial mycorrhizal inoculum was co-cultivated with maize (*Zea mays* Linn.) as the host (Watanarojanaporn et al. 2013) using Hoagland solution (full strength with low phosphate at 0.02 ppm) (Millner et al. 1992). The hosts were grown for 90 days until spores matured. The AM spores were extracted by sieve filtering (mesh sizes of 750, 250, 100, and 50 μm, respectively, from top to bottom) and centrifugation at 4000 rpm for 15 min in water and 60% (*w*/*v*) sucrose solution, respectively. The suspension was further sieved to collect the spores (Smith and Skipper 1979; Horn et al. 1993). At this point, the percentage of spore purity was approximately 50%. Finally, 25% (*w*/*v*) sucrose in water was added to the spores and unwanted particles to suspend them before loading into the microfluidic device.

Design fabrication and operation of the microfluidic device

The microfluidic device was designed to facilitate manual selection of AMF spores obtained from a spore suspension after sieving and centrifugation. It serves as a particle streaming apparatus equipped with a manual temporary flow diversion mechanism for manual single spore picking while the suspension is being streamed. The device consists of two layers: polydimethylsiloxane (PDMS) (Sylgard184, Dow Coming, MI), containing a micropattern of the device with an overall pattern height of 0.5 mm, and a glass microscope slide (75 × 25 mm) (Fig. 1a). The device was fabricated using standard UV lithography (Weibel et al. 2007) and soft lithography (Zhao et al. 1997; Konry et al. 2011; Isiksacan et al. 2016). Assembling the device was by plasma bonding (Eddings et al. 2008). The device consists of different microchannel shapes, supporting the functionality to manipulate particles within it and to separate the spores (Fig. 1b).

Spore suspensions that contained contaminating particles were injected into an inlet by syringe pump (model NE-1000, Era Pump Systems Inc., USA). The spores were directed through a funnel shaped channel (the width of the channel was 0.5–1 mm and the length was 60 mm, Fig. 1b "inlet") that helped align the particles into a single row. The spore suspension was guided to a junction to intersect sterilized water from a perpendicular channel fed by another syringe pump. At the junction, the sterilized water stream combined with the stream containing spores. The sterilized water was intended to be a guiding stream during MTFD to prevent the spores from escaping the diversion stream. When the spores arrived at the junction, the user could press a switch that would temporarily activate a solenoid valve (model LFVA1220110H, The Lee Company, USA), and in turn, generate MTFD leading single spores of interest into a collection chamber (7 mm in diameter). Non-selected, unwanted particles remained in the stream to a waste outlet.

Installation of the microfluidic device

The spore separation device was operated under a stereo microscope (model SZ621, OLYMPUS, Japan) connected to a CMOS camera to display real-time video on a computer screen. The syringe pumps were used to inject the sucrose suspension containing spores and contaminants and sterilized water (Suppl. 1).

Efficiency of spore separation using the microfluidic device versus the conventional method

The purity of separated spores and the time spent completing the task using the microfluidic device or the conventional method were compared. Ten human testers, 5 experts in the

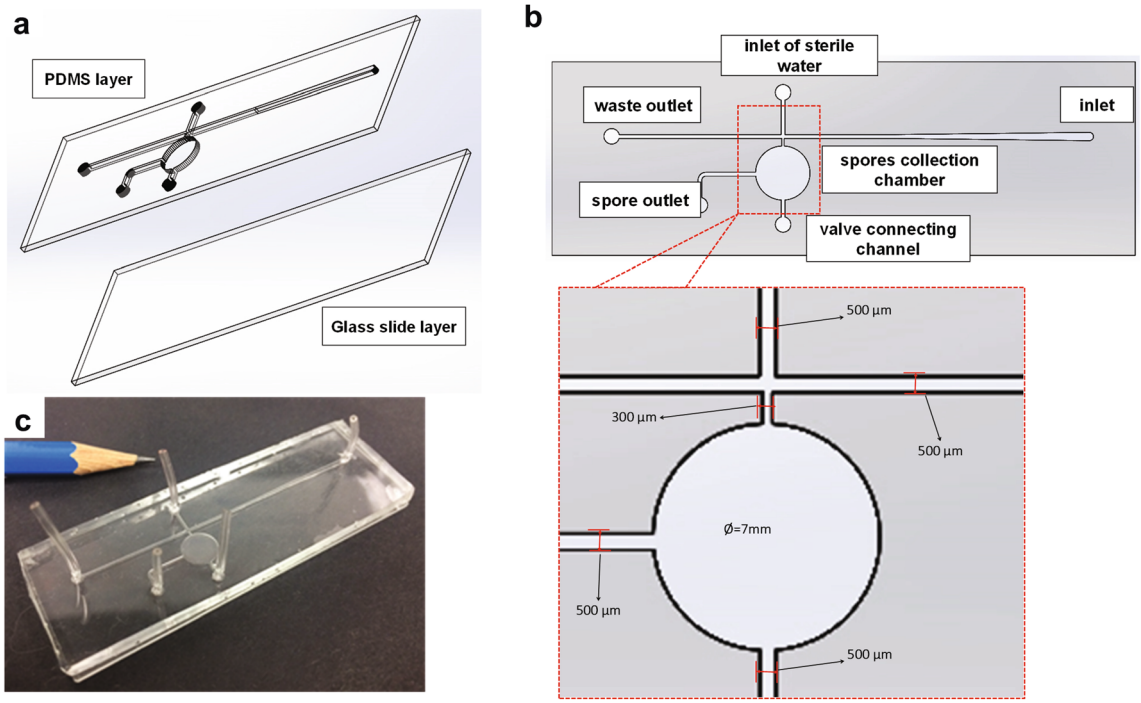

Fig. 1 The fabricated microfluidic device. **a** The device consists of 2 layers: a glass slide and the PDMS layer that embeds the channels. **b** The device comprises an inlet of spore suspension, an inlet of sterilized water, a spore collection chamber, a valve connecting channel, a spore outlet, and an outlet for waste. **c** The glass slide and the patterned PDMS layer are bound together using plasma bonding to form a complete device. The PDMS layer device is fabricated using standard UV lithography and soft lithography explained elsewhere (Weibel et al. 2007; Isiksacan et al. 2016)

field of mycorrhizal study and 5 lay persons, were asked to separate 100 spores using both methods. Prior to the experiment, the 5 lay testers received training on how to differentiate spores from contaminating particles and how to pick spores using the conventional method with a needle and micropipette. All the testers attended a practice session on how to operate the microfluidic device before the actual experiment. The practice sessions were as long as each tester required. The testers were allowed to participate in the trial only when they had reported that they were confident and comfortable at performing both separation techniques. For the microfluidic device method, sieved spores were transferred into a 25% *v*/v sucrose solution to make up a final particle concentration (C_p) of 2100 particles/mL. The spore suspension was then loaded into the device with a flow rate (R_p) of 40 μL/min. The optimal C_p and R_p were selected based on experimental results (Suppl. 2). For the conventional method, the AM spore suspension, including unwanted particles, was transferred into tap water at the optimal C_p of 2100 particles/mL in a glass Petri dish. The mycorrhizal fungus spores were separated manually under a stereomicroscope by using a needle to move them away from the unwanted particles and to group the spores. A micropipette was used to transfer the group of spores to another glass Petri dish. The number of unwanted particles found in the separated spore suspension and time spent for each method were recorded. The percentage of spore purity against the total number of particles in the collection chamber (*%SP*) was calculated. The *%SP* and the time spent were used to indicate the spore separation efficiency of each method. In our trial, all the testers performed the conventional method first and the microfluidic method second. They all had suitable rest between sessions to replenish their psychological and physical alertness before continuing with the microfluidic method.

Data analysis

To test for significant differences between the use of the microfluidic device and the conventional method, Mann-Whitney *U* tests were used at the 95% ($p > 0.05$) confidence level on the median purity of the separated spores and the median time spent using both methods.

Results

Visualization of the manual temporal flow diversion

Manual temporal flow diversion (MTFD) was employed to separate single spores in this device. To improve the separating precision with MTFD, a sterilized water stream was added as a guide flow that ran parallel to the spore suspension at the junction where the hydrodynamic flow focus was created. To visualize the flow phenomenon (Suppl. 3), yellow- and red-colored water were used to represent the spore suspension and the sterilized water, respectively. As expected, when the sterilized water stream met the stream of the spore suspension at the junction, they flowed in parallel without mixing. The hydrodynamic flow focusing phenomenon was formed without mixing (laminar mode) (Suppl. 4a). This flow pattern did not change with the alteration of R_p (i.e., 20–90 μL/min) or the corresponding flow rate of the sterilized water (double the R_p).

Suppl. 4b illustrates the interrupted flow when the valve was switched on. The MTFD was generated as a result. The steady flow pattern resumed after a few hundred milliseconds (Suppl. 4c). As seen in Suppl. 4b, in the presence of the sterilized water supply, a fraction of the sterilized water traveled to the collection chamber, temporarily blocking the flow of the spore suspension moving to the outlet.

Efficiency of spore separation using the microfluidic device versus the conventional method

In this study, the purity of the separated spores and the time spent to finish the spore separating tasks define the spore separation efficiency. The human testers ($n = 10$), 5 experienced in spore separation using the conventional method and 5 lay persons, were asked to separate 100 spores using each method. It was found that the median spore purity obtained using the microfluidic device was 96.62% ($n = 10$) and the median spore purity obtained using the conventional method was 88.89% ($n = 10$) (Fig. 2a). The median spore purity of the microfluidic method was significantly higher ($n = 10$, Mann-Whitney *U* test, $p < 0.001$) than that of the conventional method. The range of spore purity using the microfluidic device and the conventional method was 95.23 to 97.09% and 82.64 to 91.74%, respectively (Fig. 2a).

It also was found that the time spent completing the separation tasks using the microfluidic device and the traditional method were median 745.2 s ($n = 10$) and 712.2 s ($n = 10$), respectively (Fig. 2b). The time was not significantly different (Mann-Whitney *U* test, $p = 1.000$), but the range of time spent using the microfluidic device was 673.8 to 812.4 s while that of the conventional method was (90 to 1224.6 s).

Discussion

Visualization of the fluid flow within the device ensured that the manual temporary flow diversion could be properly executed using this design. Based on an experiment using a similar device, without this stream and lacking a sterilized water supply, a fraction of the spore suspension continued flowing to the outlet. As a result, there was a possibility that the targeted spores could escape separation. Additionally, when MTFD was generated in the device that lacked the sterilized

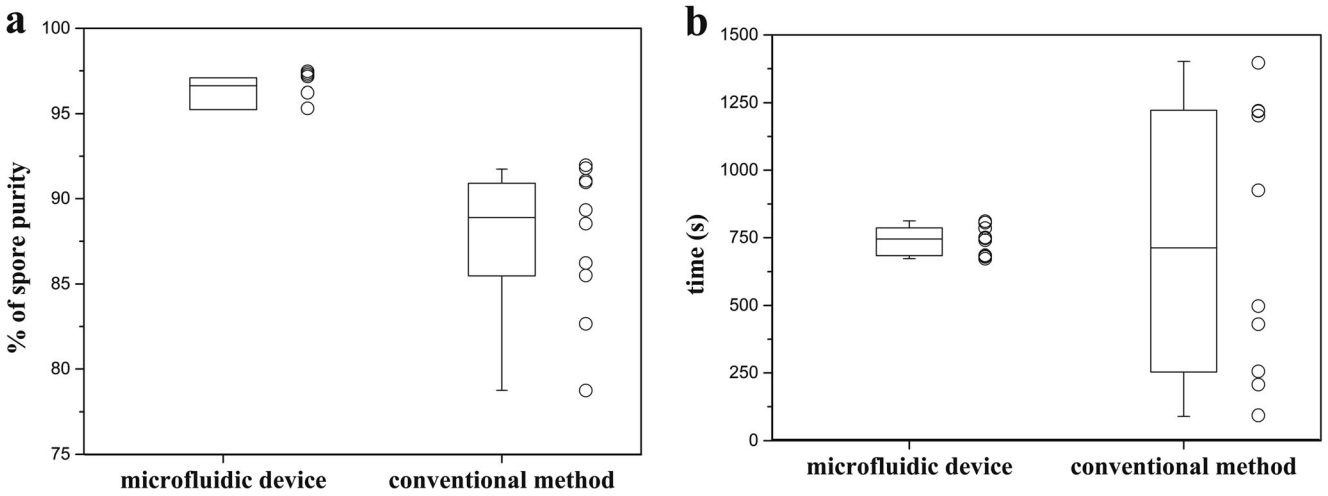

Fig. 2 The efficiency of spore separation of the fabricated microfluidic device and the conventional method. The data were based on ten subjects (5 experts in the field of mycorrhizal study, and 5 lay persons) who were asked to separate 100 spores by each method. The data points represent the subjects. The error bars represent maximum and minimum (data range). The middle lines are the median. The top and bottom of the rectangles are the third and first quartiles, respectively. **a** The median spore purity of the microfluidic method (96.62%) was significantly higher than that of the conventional method (88.89%) ($n = 10$, Mann-Whitney U test, $p < 0.001$). **b** The median time spent on spore separation using the microfluidic device (745.2 s) and the traditional method (712.2 s) were not significantly different ($n = 10$, Mann-Whitney U test, $p = 1.000$)

water supply, part of the spore suspension that flowed past the junction flowed backward and entered the collection chamber. This increased the chance of collecting unwanted particles in the collection chamber. In contrast, with our present design, there was no reverse flow spore suspension as assured by the intersecting sterilized water flow. Moreover, the sterilized water occupied about half of the combined flow channel, and around half of the volume of the sterilized water would be sucked along during the separation. As a result, the spore suspension fluid entering the spore collection channel was cut by half, with the chance of collecting unwanted particles and suspended contaminants such as bacteria reduced accordingly. The mechanism was tested with the spore suspension as shown in video online Supplement 5.

Our results show the clear advantage of the MTFD technique: it achieves a high spore purity. The significantly greater percentage of spore purity from the microfluidic device approach attests to improved spore separation. It should be noted that even the minimum spore purity obtained using the microfluidic device was still higher than the maximum obtained from the conventional method. The device allowed users to achieve a level of spore purity that was not possible with the conventional method. As reported by the human testers attempting the conventional spore picking, it was unavoidable that the micropipette picked up the group of spores without picking up unwanted particles. This could be the reason underlying the limited spore purity achieved by the conventional method. In addition, the narrowed range of spore purity using the MTFD technique indicates that any user can perform the task with consistency without the need for much prior experience or acquired skill. The approach is therefore of great use in applications where high spore purity is an objective.

The time spent to complete the task using either technique was not significantly different, but the range of time spent using the conventional method was much greater than that of the MTFD technique. The reduced variation of time spent also implies that our technique can make the spore separation less skill-dependent with respect to time consumption. Based on interviews, the participants reflected that the spore grouping and transferring under a stereo microscope using a needle in the conventional separation method were relatively challenging. It therefore took longer to complete compared with the separation procedure using the microfluidic device.

In our experiment, although the testers were asked to perform spore separation using the conventional method first and the microfluidic method second, the result nevertheless should not be biased. First, the testers had a psychological and physical reset between the first and second separations. Furthermore, performing spore separation using these two techniques requires different skill sets, so experience gained from the first separation should not help the tester to perform better in the second. In addition, because the conventional separation only took minutes to complete, skill acquisition, if any, was negligible because the testers already were proficient from their much longer training sessions (lasting several hours, or even days). The rate of improvement in skill drastically decreases once testers become skilled according to well documented skill acquisition curves (Landfried et al. 2019).

The transformation of the complex hand movement and sight coordination of the conventional method to simple

clicking for MTFD clearly simplifies separation. This may be the reason for the compressed range of efficiency compared with the conventional method. Not only can the device help refine the manipulation procedure, but it also can improve the user experience. It should be noted that a better user experience while performing the task may translate into less user exhaustion, prolonging quality working hours and improving productivity. This might help people intimidated by the skill barrier to access the field of mycorrhiza study. Thus, user interaction and ergonomics should be topics of further studies.

Based on our experimental results, the MTFD technique is applicable to AMF spore separation. Regardless of being semi-automatic, it eases the separation of known target spores from highly diverse groups of uncharacterized particles. This is one of the main differences from previously developed cell separation techniques that usually deal with samples of mixed known particles of low character diversity such as separation of human mesenchymal stem cells (hMSC) from osteoblasts (Song et al. 2015) and parasites from human blood (Holm et al. 2011).

Our preliminary work demonstrated that the MTFD technology has the potential to be implemented in multiple applications, especially for improving the purity of inocula and the separation of spores of interest from mixed spore samples. In our experiments, the separation worked well with spores of a single species collected from a pot culture with sand and vermiculite as the substrate. One of the challenges and a next step towards further technological development of the application is to broaden the sample types to mixtures of spores of several fungus species in field soils, clayey soils, and in substrates that contain a high amount of organic matter which have proven to be challenging for spore extraction (Allen et al. 1979; Ianson and Allen 2013). Study of the pretreatment of the sample before separation such as with Calgon (Allen et al. 1979) to clean up the spore mixtures is needed.

It is well known that the AMF used in propagation and research come in various forms such as hyphae, root fragments, and spores that may be attached to collapsed saccules. Although the present prototype was mainly developed for use with globose, single spores, a similar mechanism could be adapted for use with other forms and sizes of AMF spores. In those cases, further optimization of channel sizes, valve opening times, and other associated factors must be considered.

Our technology was developed based upon spores being suspended in a sucrose solution during the separation. The sucrose solution helps improve spore buoyancy in the device but changes the osmotic pressure of the spore suspension. This could be an issue in work where spore vitality and infectivity are of concern. The development of a sucrose-free variation is another step to develop this technology.

Based on our experimental results, using the optimized flow rate and particle concentration, up to 0.7–1.0 ml of the spore suspension can be processed resulting in the collection of 500 spores per hour. The proportion of spores lost during separation using the device was approximately 10%. Improvement of the technology's efficiency is a topic for future studies. Nevertheless, this work opens the door to possible automation. By incorporating image processing and machine learning, spore separation based on visible characteristics of spores such as shape, size, and color might be performed faster and more efficiently than even with MTFD. It additionally may be possible not only to improve inoculum purity but to sort spores based on taxa, age, and quality.

Conclusion

In this research, we successfully developed a microfluidic device for manual spore separation based on MTFD. The technology helps to significantly improve the efficiency of spore separation and is more user-friendly than the conventional method. The efficiency of the method can be further improved, and it could be developed into a fully automated system, with the help of image processing and machine learning.

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