

# Characterizing and handling different kinds of AM fungal spores in the rhizosphere

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**Abstract** Spores are important propagules as well as the most reliable species-distinguishing traits of arbuscular mycorrhizal (AM) fungi. During surveys of AM fungal communities, spore enumeration and spore identification are frequently conducted, but generally little attention is given to the age and viability of the spores. In this study, AM fungal spores in the rhizosphere were characterized as live or dead by vital staining and by performing a germination assay. A considerable proportion of the spores in the rhizosphere were dead despite their intact appearance. Furthermore, morphological and molecular analyses of spores to determine species identity revealed that both viable spores and dead spores with contents were identified. The accurate identification of spores at different developmental stages on the basis of morphology requires considerable experience. Our findings suggest that surveys of AM fungal communities based on spore enumeration and morphological and molecular identification are likely to be inaccurate, primarily because of the large proportion of dead spores in the rhizosphere. A viability check is

recommended prior to spore molecular identification, and the use of trap cultures would give more reliable morphological identification results. We show that the abundance and activity of AM fungi in the rhizosphere can be determined by calculating the density of viable spores and the density of spores that could germinate. The adoption of these methods should provide a more reliable basis for further AM fungal community analysis.

**Keywords** Spore density · Spore germination · Spore identification · Spore viability

## Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous in most ecosystems and form a symbiotic association with the roots of more than 80 % of terrestrial plants (Smith and Read 2008). AM fungi are obligate biotrophs and, hence, can only fulfill their life cycles by obtaining carbohydrates from a host. Generally, there are three kinds of AM fungal propagules present in soils: spores, extraradical mycelium and infected root segments (Druille et al. 2013; Smith and Read 2008). Extraradical mycelium is considered to be the most important source of inoculum among the species belonging to the Glomeraceae (Schalamuk and Cabello 2010). By contrast, spores are the main source of inoculum among species belonging to the Gigasporaceae because these AM fungi colonize roots primarily from spores (Schalamuk and Cabello 2010).

Asexual AM fungal spores are transiently dormant, persistent propagules that remain infectious in the absence of host plants and can survive under unfavorable conditions (Klironomos and Hart 2002). Spores represent an important feature of the life history of AM fungi, and their isolation

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and quantification is a relatively straightforward process and accessible for most researchers. Till now, the morphological characteristics of these largely soil-borne spores have remained the main species-distinguishing traits of the Glomeromycota (Oehl et al. 2009).

During AM surveys in natural ecosystems, usually a series of works need to be done on the AM fungal spores, among which, spore enumeration (spore density, SD) and spore identification (mainly by morphological analysis) are most frequently conducted. Spore density is important for revealing the abundance of AM fungi in the rhizosphere, which is used to determine the AM fungal infective potential in the rhizosphere and for determining the activities of the AM fungi colonizing the roots (Abbott and Robson 1991; Castillo et al. 2006). Although the “Most Probable Number” method can be used to determine the number of infective propagules of AM fungi (Porter 1979), this method is time-consuming and, therefore, infrequently used. Spore identification, which is common practice in studies of AM fungal ecology, is essential for characterizing the AM fungal community (Oehl et al. 2005; Smith and Read 2008). However, in practice, there has been little awareness of the different kinds of AM fungal spores in the rhizosphere and their effect on the accuracy of the results of spore enumeration and spore identification.

AM fungal spores in the rhizosphere can be categorized as live or dead spores or categorized on the basis of their different growth stages: that is, as juvenile, mature or old spores. Dead spores may persist in soil for extended periods (McGraw and Hendrix 1986), and it is difficult to discriminate dead spores from viable spores based on appearance (Lee and Koske 1994). The inclusion of dead spores in calculations of spore density, particularly when dead spores make up a high proportion of the total spores, is likely to provide an erroneous impression of spore density. Furthermore, morphological and molecular identification of dead spores, particularly those with totally exhausted contents, is a waste of time and resources. Evaluation of spore viability would be an efficient way of excluding dead spores; however, evaluations of spore viability have seldom been conducted. Spores at different developmental stages (which usually have distinct morphological features, such as size and color) may also hinder spore morphological identification, which is based on the morphological features of the spore, such as size, color, and the layers of the cell wall (Brundrett et al. 1996).

In this study, we investigated the density of different kinds of AM fungal spores (categorized by spore viability) in the rhizosphere of five plant species growing in different substrates. The possible impact of dead spores on the identification of AM fungi was also evaluated using monocultures of AM fungi.

## Materials and methods

### Spore density of different kinds of spores (categorized by spore viability) in the rhizosphere

#### *Ethics statement*

The locations of the field studies are neither private lands nor protected areas and are controlled by the Management Committee of Northwest A&F University or by the Forestry Administration of Hengshan County. Both the Management Committee of Northwest A&F University and the State Forestry Administration authorized us to conduct root/soil sampling in the regions that they control. Field studies did not involve endangered or protected species according to Chinese laws.

#### *Rhizospheric substrate sampling and AM fungal spore collection*

In October 2008, rhizospheric substrates were collected from the fine roots of at least five plants of five plant species: *Trifolium repens* L. (white clover), *Zea mays* L., *Robinia pseudoacacia* L., *Populus simonii* Carr., and *Caragana korshinskii* Kom. (see Table 1 for details). The available N, P and K and the organic matter in each of the substrates were determined (Bao 2000).

Spores were extracted from 100 g of air-dried substrate of each sample using a wet sieving and decanting method (Gerdemann and Nicolson 1963), with five biological replications. To maximize spore viability we did not use the sucrose gradient method (Walker et al. 1982) to collect the relatively clean AM fungal spores because this method has been shown to be detrimental to spore viability (Salvador-Figueroa et al. 2008). All apparently healthy-looking spores were collected and counted under a stereomicroscope. Spores were stored at 4 °C for at least 2 weeks (to break dormancy) prior to use (Juge et al. 2002). However, not all AM fungal species have a period of spore dormancy (Giovannetti 2000), and in some species spore viability is affected by cold storage. To determine whether cold storage did affect the viability of the spore communities under investigation, we also included a control treatment in which the spores were not subjected to a period of cold storage.

#### *Spore viability evaluation and spore germination assay*

The proportions of four kinds of spores (dead spores with contents exhausted, i.e. empty spores; dead spores with contents; viable spores that could not germinate; spores that could germinate) in the rhizosphere were investigated

**Table 1** Characteristics of the rhizospheric substrates

Host plant	Growth substrate <sup>a</sup>	Available N (μg/g)	Available P (μg/g)	Available K (μg/g)	Organic matter (%)	pH
<i>Trifolium repens</i>	Lou soil <sup>b</sup>	45.18 ± 5.79	16.98 ± 0.89	193.30 ± 11.24	1.05 ± 0.09	7.43 ± 0.33
<i>Zea mays</i> <sup>d</sup>	Fine sand	–	–	–	–	–
<i>Robinia pseudoacacia</i>	Cultivated loessial soil <sup>c</sup>	35.83 ± 8.17	3.29 ± 0.77	73.99 ± 10.35	0.68 ± 0.11	7.83 ± 0.45
<i>Populus simonii</i>	Loessial sandy soil <sup>c</sup>	25.69 ± 3.44	3.05 ± 0.36	62.71 ± 8.97	0.79 ± 0.21	7.67 ± 0.12
<i>Caragana korshinskii</i>	Aeolian sandy soil <sup>c</sup>	22.46 ± 5.13	3.31 ± 0.53	74.70 ± 7.62	0.99 ± 0.09	7.67 ± 0.09

Data are mean ± SE;  $n = 5$

<sup>a</sup> The lou soil, cultivated loessial soil, loessial sandy soil and aeolian sandy soil are typical soil types of the Shaanxi Province of China

<sup>b</sup> Collected from the campus of Northwest A&F University (34°27'N; 108°07'E; 453 m altitude)

<sup>c</sup> Collected from state-owned forest farms located in Hengshan County, Shaanxi Province (37°85'N; 109°46'E; 1050 m altitude)

<sup>d</sup> *Z. mays* plants were initially inoculated with *Funneliformis mosseae* and supplied with modified Hoagland solution (containing 19.40 mM N, 20 μM P and 2 mM K; pH = 6.00) once a week (50 mL per pot) during the growth period

using 100 randomly selected spores per sample. Spore viability was determined using the tetrazolium chloride vital stain INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride] following the procedure used by Walley and Germida (1995). INT has previously been shown to be more reliable as a rapid indicator of AM fungal spore viability than the tetrazolium bromide vital stain MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] (Walley and Germida 1995). Spore suspensions were diluted 1:1 with a solution of 2 mg/mL INT (to reach a final concentration of 1 mg/mL) and incubated at 28 °C for 72 h. Spores with contents that stained red were considered viable (spores were crushed in case the colors of some spores were too dark to observe). Spores that did not stain red were considered dead and were crushed with a fine needle to determine whether they had any contents (to calculate the ratio of empty spores).

One hundred randomly selected spores per sample were surface sterilized (Bécard and Fortin 1988) prior to incubation on 0.6 % plant agar medium at 25 °C for 2 weeks. Given that bacterial and fungal contaminations may persist after the surface sterilization process, each Petri dish was only inoculated with ten spores. The frequency of bacterial and fungal contaminations was recorded at the end of the experiment. A control experiment that lacked the surface sterilization step was conducted to evaluate the possible influence of sterilization agents on spore germination; however, no significant differences in germination rate were found compared with those of sterilized spores (data not shown).

The density of viable spores and the density of viable spores that could germinate were calculated by multiplying the spore density with the corresponding spore ratio.

#### AM colonization evaluation

The root samples were cleared and stained (Phillips and Hayman 1970) and the proportion of the root colonized by

AM fungi was determined using a magnified intersections method (McGonigle et al. 1990) with 150 randomly selected root segments (1 cm in length; Sun and Tang 2012).

#### Interference of different kinds of spores on spore identification

To evaluate the impact of dead spores with contents on the spore identification procedure, we collected spores of *Funneliformis mosseae* (formerly *Glomus mosseae*) BGC NM01A propagated in a pot-culture stored at room temperature for more than 1 year. To avoid the influence of juvenile spores on spore identification (particularly morphological identification), only those spores with a diameter larger than 100 μm were chosen. A viability assay was conducted using INT as the vital stain (the same procedure described above). Spores with contents that stained red were considered viable, and otherwise, spores were considered dead. After the viability assay, several spores (both dead spores and viable spores) were crushed for morphological identification [according to the procedure described by Oehl et al. (2010)] or for single-spore molecular identification (Nested PCR: first round primers, NS1 and NS4; second round primers, AML1 and AML2. The size of the target DNA segment was 800 bp; Lee et al. 2008). For dead spores, we mainly focus on those with contents, and after crush, the dead spores without contents were discarded without further morphological or molecular analysis. For morphological identification, spores were selected under the dissecting microscope and mounted in polyvinyl alcohol-lactic acid-glycerine (PVLG) or PVLG mixed 1:1 (v:v) with Melzer's reagent for semi-permanent slides. The spores were then examined using a compound microscope at up to 400-fold magnification. Species identification was based on current species and identification manuals [Schenck and Pérez 1990; International Culture Collection

of (Vesicular) Arbuscular Mycorrhizal Fungi (<http://invam.wvu.edu/>).

## Data analysis

Data were statistically analyzed by one-way ANOVA using the statistic software SPSS 17.0.0 (Statistical Product and Service Solutions, SPSS Inc. Chicago, IL). The mean values of samples were compared using the Duncan multiple range test ( $P < 0.05$ ). Data are presented as means and SE.

## Results

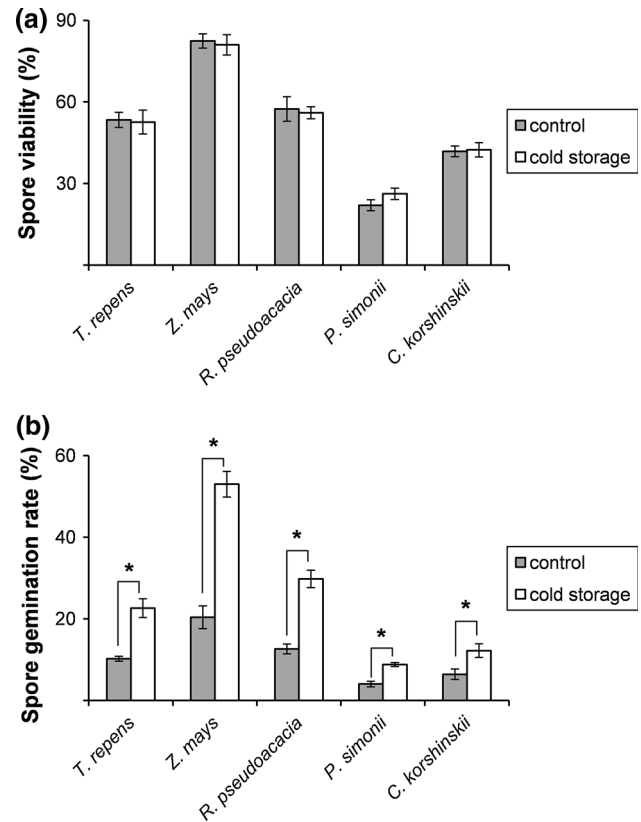
### Spore density of different kinds of AM fungal spores (categorized by spore viability) in the rhizosphere

All five plant species were well colonized by AM fungi: the colonization rates were  $61.23 \pm 3.59\%$  (*T. repens*),  $56.69 \pm 1.05\%$  (*Z. mays*),  $63.65 \pm 1.68\%$  (*R. pseudoacacia*),  $61.29 \pm 1.36\%$  (*P. simonii*) and  $45.49 \pm 3.12\%$  (*C. korshinskii*). The spore densities in the rhizospheres of the five plant species were  $118 \pm 12$  (*T. repens*),  $1484 \pm 121$  (*Z. mays*),  $1427 \pm 73$  (*R. pseudoacacia*),  $170 \pm 11$  (*P. simonii*) and  $160 \pm 9$  (*C. korshinskii*) per 100 g dry substrate.

Cold storage (4 °C) had no effect on spore viability, but significantly improved the spore germination rate (Fig. 1). Although we cannot guarantee that cold storage did not have a negative effect on any of the species, cold storage appeared to be an efficient method of improving the germination rate of the whole AM fungal community.

The proportion of dead spores (both with and without contents) in the rhizosphere of *P. simonii* and *C. korshinskii* was greater than the proportion of viable spores (Fig. 2a). The proportion of viable spores was greater than the proportion of spores that could germinate in all the rhizospheres examined: for example, in the *T. repens* rhizosphere there were 2.59 times as many viable spores as spores that could germinate. Spores from the AM fungal monospecific culture (*T. repens*) had a greater viability ( $80.60 \pm 3.96\%$ ) and germination rate ( $54.20 \pm 4.03\%$ ) because most of the spores were newly formed, which is consistent with the findings of an earlier study (An et al. 1998). Although the spore densities and soil properties of the *P. simonii* and *C. korshinskii* rhizospheres were similar (Table 1; Fig. 2b), the proportion of viable spores present was significantly different ( $22.80 \pm 2.89\%$  for *P. simonii* and  $41.60 \pm 3.01\%$  for *C. korshinskii*;  $P < 0.05$ ), indicating that the spore densities in the rhizospheric substrates of the different plant species were not comparable.

Spores collected from plant rhizospheres were comprised of spores of various AM fungal species with distinct



**Fig. 1** Effects of cold storage on **a** spore viability and **b** the spore germination rate. Bars show the SE;  $n = 5$ . Significant differences in the spore germination rate are indicated by an asterisk ( $t$  test,  $P < 0.05$ )

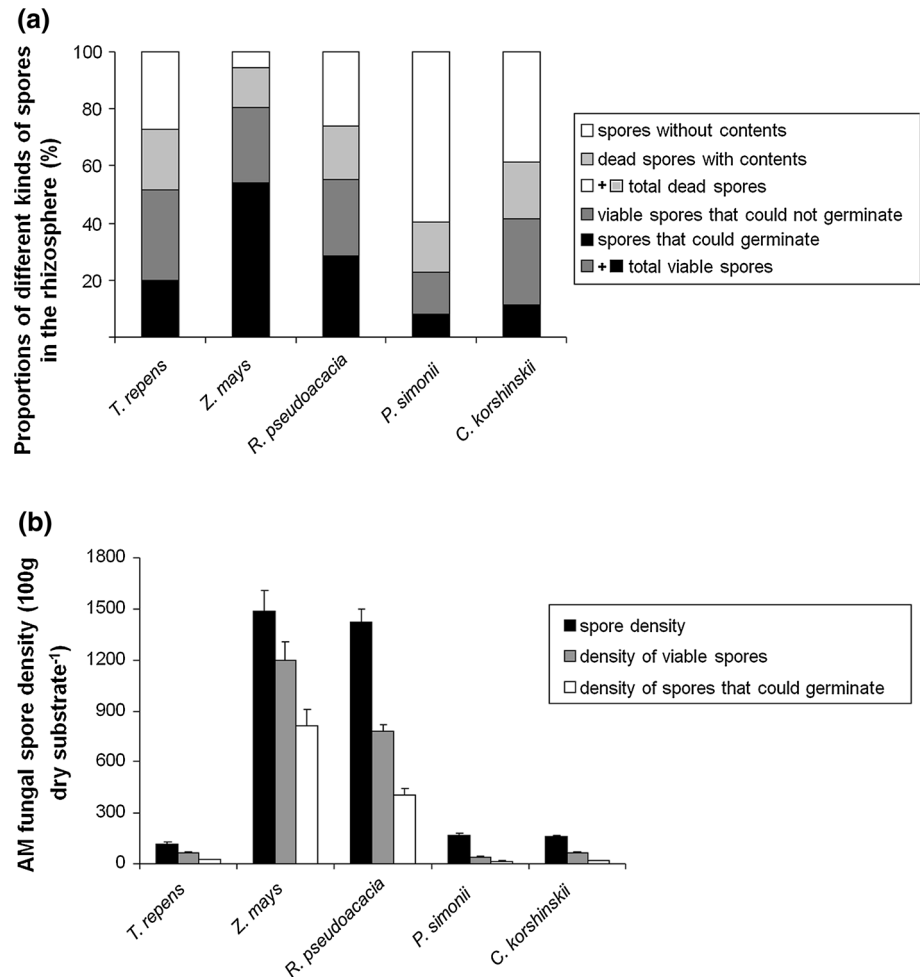
morphological characteristics. Some germinated spores from different AM fungal species are shown in Fig. 3.

Even after surface sterilization, there were still some spores that were contaminated by bacterium or fungi. The proportion of contaminated spores among the germinated spores and spores that cannot germinate was similar (Fig. S1). It is unclear whether spore germination was affected by microbial contamination in the present study.

### Interference of different kinds of spores on spore identification

The dead spores of *F. mosseae* with contents were successfully identified using both morphological and molecular identification methods (Fig. 4). INT staining did not affect the molecular analysis of viable spores but did affect their morphological features (Fig. S2). The contents of viable spores were stained red and the spore wall became transparency after the permeation by the INT stain. The size of the viable spores ( $124 \pm 12 \mu\text{m}$ , mean diameter;  $n = 100$ ) and of the dead spores ( $119 \pm 13 \mu\text{m}$ , mean diameter;  $n = 100$ ) was not significantly different.

**Fig. 2** AM fungal spores in the rhizosphere categorized by viability and whether they have content. **a** Proportions of different kinds of spores in the rhizosphere; **b** spore densities of different kinds of spores in the rhizosphere. Bars show the SE;  $n = 5$



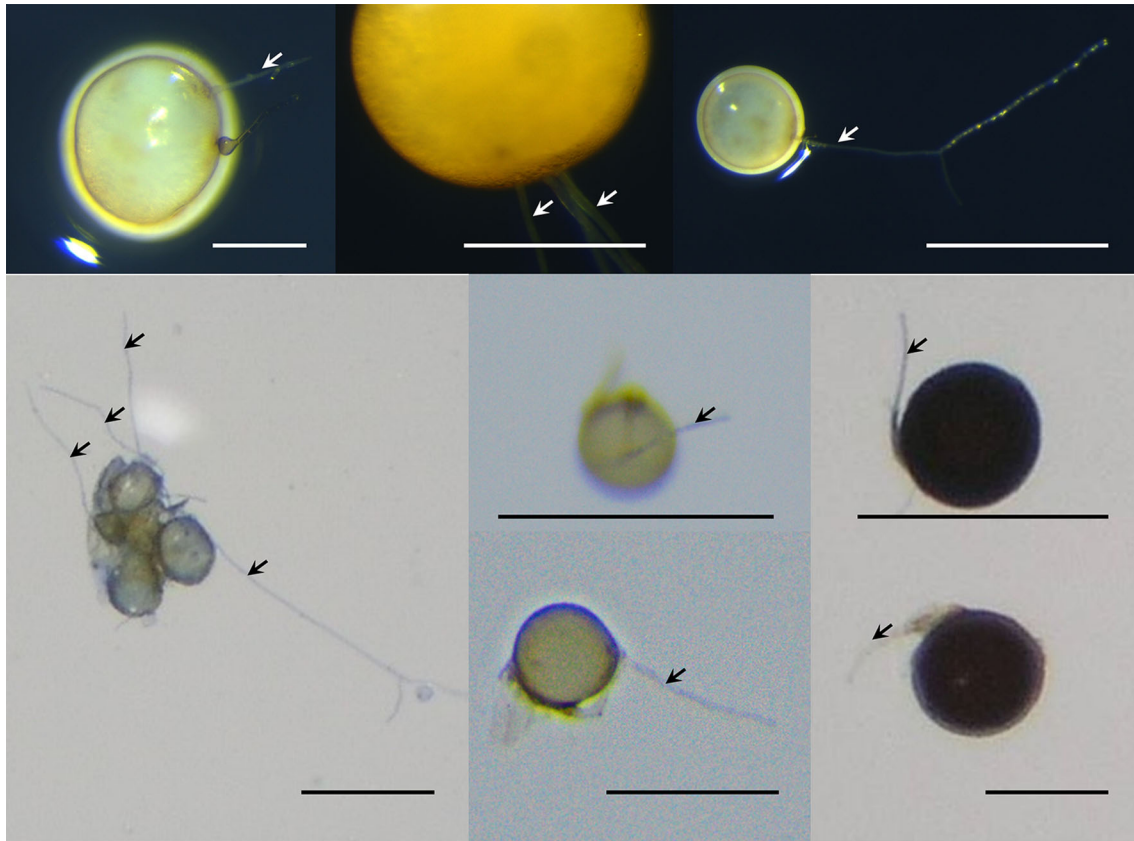
## Discussion

Understanding the roles of AM fungi in the biology of their hosts must include understanding the roles of spores in the biology of the fungi (An et al. 1998). Usually, a survey of AM fungal spores involves spore enumeration and spore identification, which are influenced by the composition of the spore communities (the age and viability of spores).

In the present study, we found that AM fungal spores in the rhizosphere can be divided into four kinds based on their viability. The proportion of dead spores (both with and without contents) in the rhizosphere of *P. simonii* and *C. korshinskii* was greater than the proportion of viable spores, which may explain why single spore propagations always have a low rate of success. There were considerable numbers of dead spores with an intact appearance in the rhizospheres of the five different plant species growing in different substrates that could not be distinguished from viable spores, thus spore density correlated poorly with mycorrhiza formation and/or activity. Furthermore, there are other more infective propagules (Porter 1979), such as

intraradical mycelium, and there are also some species that never sporulate (Baylis 1969). Indeed, spore density mainly reflects the accumulated sporulation history of the respective soil (Hijri et al. 2006). Without knowing the number of viable spores and whether those spores could germinate, it is impossible to further investigate the presence and richness of a specific AM fungal species in the rhizosphere and to evaluate their potential as propagules (An et al. 1998; Liu and Luo 1994).

The empty spores with a healthy appearance that were present in the rhizosphere may have already germinated and have exhausted their contents. These spores cannot be identified by morphological or molecular methods, and, hence, they would not affect the results of an investigation of the active AM fungal community; however, time spent identifying dead spores with exhausted contents is a waste of time and resources. There were also dead spores with contents present in the rhizosphere (it is possible that these may have been live spores that were damaged during soil sample collection and or later during spore isolation or spores that died for

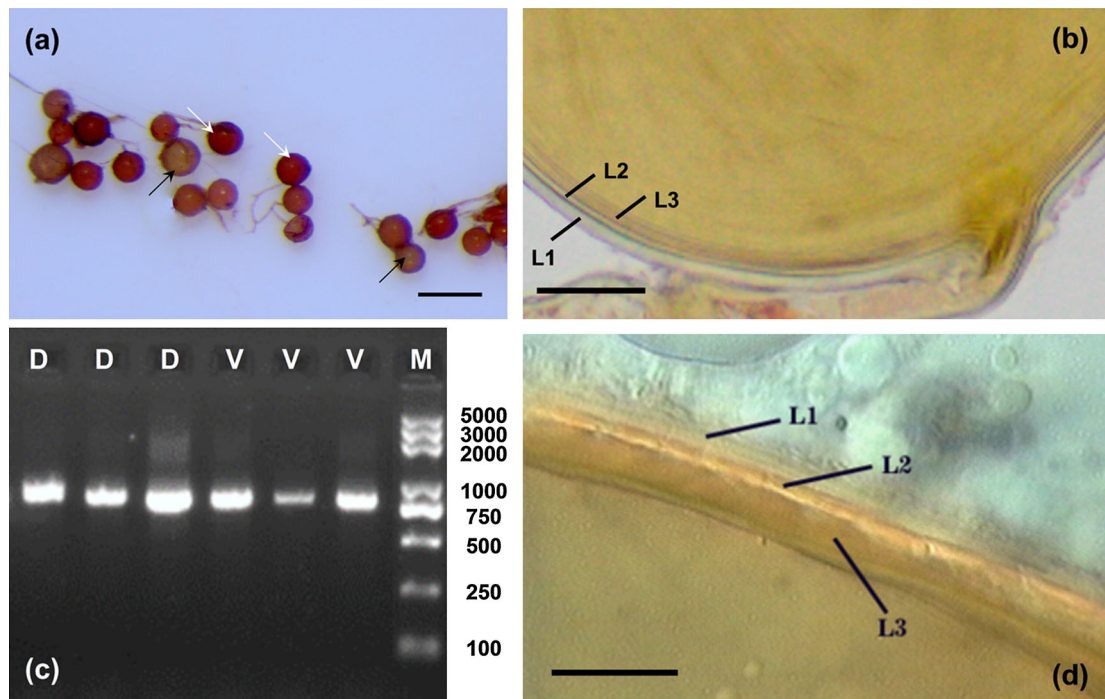


**Fig. 3** Germinated spores with distinct morphological characteristics. Germ tubes are indicated by *arrowheads*. *Scale bars* represent 200  $\mu\text{m}$

other unknown reasons). These spores should be excluded when estimating the activity or infective potential of the AM community. However, whether these dead spores should be considered during an AM fungal community investigation is debatable. Our results revealed that these dead spores with contents could be successfully identified by both morphological and molecular identification methods, which would affect the conclusions drawn about the composition of an AM fungal community in the rhizosphere. It is intriguing to find that the INT treatment did not affect the molecular analysis of spores but impact the morphological identification of viable spores. We propose that a viability survey is necessary to select the effective (viable) spores for molecular identification.

The viable spores and spores that could germinate are the most interesting and could indicate the potential of spores as propagules (Druille et al. 2013). The proportion of viable spores was always greater than the proportion of spores that could germinate. It is possible that spores that do not germinate the first year following formation may persist in the soil for several years without losing viability (McGraw and Hendrix 1986).

Changes in spore viability may lead to changes in AM fungal diversity, AM fungal population dynamics and in the functionality of the symbiosis given the importance of spores as a source of propagules for the perpetuation and spread of AM fungi in the ecosystem and for the optimal root colonization of plants (Smith and Read 2008). To obtain a more reliable estimation of AM fungal propagules in the rhizosphere, we used two indicators: density of viable spores and density of spores that could germinate. The density of viable spores represents the quantity of spores that should be examined during AM fungal community analysis and is most likely correlated with mycorrhizal formation and/or activity in roots (these viable spores are most likely the newly formed spores). By contrast, the density of spores that could germinate directly represents the quantity of active spore propagules in the rhizosphere, which should be considered during the propagation of AM fungi, particularly those that propagate using spores. We suggest that during surveys of AM fungi, spore density, density of viable spores and density of spores that could germinate should be considered simultaneously to obtain a more reliable estimation of the abundance and activity of AM fungi in the rhizosphere as



**Fig. 4** Viability assay and identification of *Funneliformis mosseae* spores. **a** Spores stained with 0.1 % INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride] for 72 h. *Black arrowheads* indicate dead spores; *white arrowheads* indicate viable spores. The *scale bar* represents 200  $\mu$ m. **b** Spore wall of dead spore. *L1*, *L2*, and *L3* indicate three different layers of the spore wall. The *scale bar* represents 20  $\mu$ m. **c** The second round of PCR amplification

of a single spore of *F. mosseae* using the primer pair AML1 and AML2. *D* dead spore stained with INT, *V* viable spore stained with INT, *M* DNA ladder. **d** Spore wall of a viable *F. mosseae* spore (the image was taken from the INVAM website: <http://invam.wvu.edu/the-fungi/classification/glomaceae/funneliformis/mosseae>). *Scale bar* represents 10  $\mu$ m

well as to provide a more reliable basis for further AM fungal community analysis.

Morphological analysis results in a more comprehensive differentiation within a specific AM fungal genera compared with molecular analysis (Wetzel et al. 2014); however, the drawback is that the person performing the morphological analysis must be able to recognize the juvenile, mature and old spores of each species. These different kinds of spores have distinct morphological features (e.g. size and color), which can make morphological analysis a challenge for those with limited experience of morphological identification. To avoid potential misidentification, which could lead to erroneous impressions of the AM fungal community, trap cultures are recommended prior to morphological analysis (Gai et al. 2006; Oehl et al. 2005, 2009). However, according to our literature survey of 300 related research studies, only 16 % of these studies conducted trap cultures. Although trap cultures are unsuitable for characterizing AM fungal communities from field soils because they do not reflect the in situ reality (Säle et al. 2015), it is claimed that under long-term growth conditions and using a soil pH and climate as close as possible to natural conditions, trap cultures would largely re-establish AM fungal communities (Rosendahl 2008),

and furthermore, they could largely avoid the bias associated with the inclusion of ecologically inactive resting spores in whole soil samples.

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