Soil spore bank in Tuber melanosporum: *up to 42% of fruitbodies remain unremoved in managed truffle grounds*

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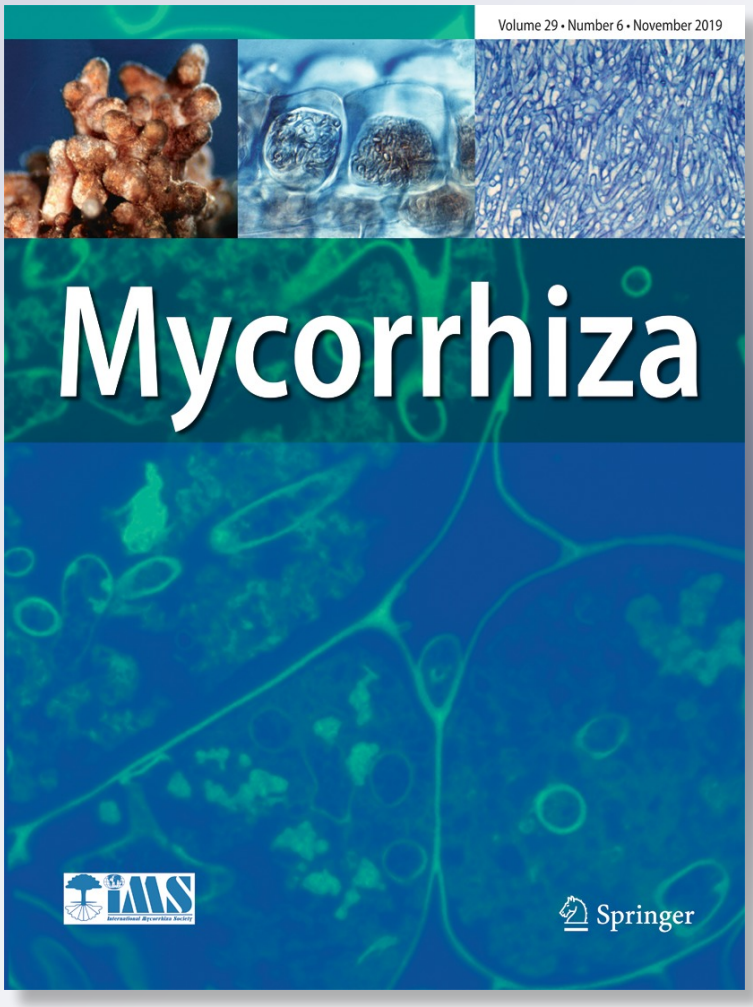
Mycorrhiza

ISSN 0940-6360
Volume 29
Number 6

Mycorrhiza (2019) 29:663-668
DOI 10.1007/s00572-019-00912-3



Springer

Mycorrhiza (2019) 29:663–668
https://doi.org/10.1007/s00572-019-00912-3

SHORT NOTE

Soil spore bank in *Tuber melanosporum*: up to 42% of fruitbodies remain unremoved in managed truffle grounds

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Received: 18 May 2019 / Accepted: 12 August 2019 / Published online: 7 November 2019

Abstract

Fungi fruiting hypogeously are believed to form spore banks in soil especially because some fruitbodies are not removed by animals. However, little is known on the proportion of fruitbodies that are not removed by animals. We took advantage of the brûlé phenomenon, which allows delineation of the mycelium distribution, to assess the proportion of unremoved black truffle (*Tuber melanosporum*) fruitbodies in the context of plantations where fruitbodies are actively sought and harvested by truffle growers. We inspected portions of the brûlés after the harvest season to find unremoved fruitbodies. On average, from six truffle grounds in which a total of 38 brûlés were investigated, unremoved fruitbodies represented 33% of the whole fruitbody production (42% when averaging all the brûlés). We discuss this value and its high variability among truffle grounds. Beyond the local and variable accidental reasons that may lead to this high proportion, we speculate that the formation of some undetectable fruitbodies may be under selection pressure, given the reproductive biology of *T. melanosporum*.

Keywords Ascomycetes life cycle · Brûlé · Mycorrhizae · Spore dispersal

Introduction

The existence of a spore bank in soil is well characterized in fungi forming hypogeous fruitbodies (e.g., Kjøller and Bruns 2003; Bonito et al. 2012; Glassman et al. 2015; Séne et al. 2018). In such species, animals usually disperse spores by ingesting the fruitbodies (Urban 2017; Vašutová et al. 2019), and a part of the spore bank is deposited by feces, but another part may arise from fruitbodies that are not removed by animals and remain in the soil. The spores left in soil survive well, especially compared with those of fungal species that disperse spores aerially (Bruns et al. 2009; Murata et al. 2017), likely due to the thick wall adapted to protect them in the digestive tract of animal dispersers. Germination of this spore bank may lead to settlement of new individuals, genetically related to the existing parents. This results in a pattern where spatially close individuals are genetically close, i.e., a pattern of isolation by distance (IBD; for a review, see Douhan et al. 2011; Vincenot et al. 2017; Schneider-Maunoury et al. 2018), as characterized in *Rhizopogon* spp. (Kretzer et al. 2005; Dunham et al. 2013) or in *Tuber melanosporum* (Bertault et al. 2001; Taschen et al. 2016; de la Varga et al. 2017). In the current short note, we investigate the occurrence of unremoved fruitbodies of the highly prized black truffle, *Tuber melanosporum*, under managed conditions where ground managers extensively harvest fruitbodies.

The biological relevance of the spore bank is very important for *T. melanosporum* because spores are believed to have an additional gametic role in this and likely other *Tuber* species. Fruitbody formation results from a mating between two individuals of different mating types (Selosse et al. 2017). The maternal individual forms the sterile tissues of the fruitbody

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00572-019-00912-3) contains supplementary material, which is available to authorized users.

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and likely supports and feeds fruitbody development, while the paternal individual only provides genes that can be found in the meiotic ascospores (Riccioni et al. 2008; Murat et al. 2013; for review, see Selosse et al. 2017). Maternal individuals colonize surrounding trees as ectomycorrhizal partners (Riccioni et al. 2008; Taschen et al. 2016). Moreover, in the brûlé, i.e., the zone around host trees where *T. melanosporum* develops and where herbaceous plants grow poorly (Streiblová et al. 2012), maternal individuals colonize as endophytes the roots of non-ectomycorrhizal plants (Schneider-Maunoury et al. 2018, 2019). Conversely, paternal individuals are found neither on ectomycorrhizal roots (Murat et al. 2013; Taschen et al. 2016) nor as endophytes in non-ectomycorrhizal plants from the brûlé (Taschen et al. 2016; Schneider-Maunoury et al. 2018, 2019). However, the possibility that paternal individuals live far away is not plausible because of the high consanguinity between male and female in each fruitbody (Taschen et al. 2016; de la Varga et al. 2017), which, given the existence of an IBD pattern, implies that both parents occur in close vicinity. The apparent absence of vegetative presence of paternal individuals prompted the hypothesis that they were germinating spores (Selosse et al. 2013; Taschen et al. 2016). Spores may therefore have a gametic contribution to fruitbody formation in *T. melanosporum*.

Under this assumption, the *T. melanosporum* spore bank is not only a local inoculum providing new mycelia, but also a crucial resource for future matings and the subsequent formation of fruitbodies. Yet, the active fruitbody harvest for commercial reasons may preclude a sufficient spore bank in managed truffle grounds. This may even explain why the large-scale inoculations and plantations in France (now accounting for 80% of the harvest; Murat 2015) did not reverse the ca. 10-fold reduction of the truffle production since the beginning of the twentieth century (Callot 1999; Le Tacon 2017). One may question whether truffle harvesting in truffle grounds, based on weekly detection of ripe fruitbodies by trained dogs or less often pigs (Callot 1999), may limit the spore bank. Here, we estimate the percentage of unremoved fruitbodies at the end of the fruiting season on productive brûlés.

Material and methods

Choice of brûlés

We asked French truffle growers to choose brûlés of roughly circular shape for which they knew the number F of fruitbodies produced over the previous harvesting season (fall to winter). The crucial point in this study is that the brûlé allows spatial delineation of the mycelium distribution. Assuming a circular shape (and avoiding too irregular brûlés), we were able to approximate the surface S of these brûlés. In this report, we only considered truffle grounds with more than 3 investigated brûlés. All truffle grounds are managed plantations sensu domesticated situation in Taschen et al. (2016).

Detection of unremoved fruitbodies

At the end of the fruiting season in 2018 or 2019 (late winter/early spring), depending on the truffle ground (Table 1), at a time when dogs no longer detect any fruitbodies, 4 wells of 30 × 30 cm were opened on each brûlé (Figure S1a,b). These limited areas minimized brûlé disturbance. The wells were situated on the brûlé at random distances and random orientations from the tree trunk. The soil was excavated down to the maximal depth where the truffle growers usually harvest fruitbodies in their truffle ground (20 cm at least, and more according to soil type): our record is conservative, since we cannot exclude that additional fruitbodies occur deeper. This means investigating a surface of $s = 4 \times 0.3 \times 0.3 = 0.36$ m^2 on each brûlé. The number f of fruitbodies visually removed (i.e., without dog and not using smell for detection) is recorded. We estimate the percentage of unremoved fruitbodies by extrapolating the number of unremoved fruitbodies to the whole brûlé, and by dividing by the observed production, i.e., as $100 \times (f/S)/(F/S + f/s)$.

Results and discussion

Unremoved fruitbodies

We analyzed 38 brûlés from six truffle grounds that produced 1 to 56 fruitbodies during the year of investigation, which means that 152 wells were realized (Table 1). Each of these wells revealed 0 to 6 unremoved fruitbodies, i.e., in all 0 to 8 fruitbodies per brûlé (Table 1). They displayed various states of preservation (but were all ripe to overripe; Figure S1c), and occurred at various depths (although this was not quantified, several fruitbodies were even very close to the surface). Since the search was conducted at depths where fruitbodies are normally collected on the respective truffle ground, we can exclude the detection of fruitbodies that were missed by dogs because of a too deep location. We consider unlikely that these fruitbodies were from previous years because (i) the high biological activity in the mull-type soils of truffle ground would not allow this and (ii) the monitoring of soil-implanted fruitbody pieces reveals full dismantlement and dispersal by macrofauna within 2–3 weeks (Barry-Etienne, Jourdan & Murat, personal communication). Some fruitbodies may even have disappeared at the time of sampling. Thus, we offer a conservative estimate of the number of unremoved fruitbodies, i.e., additional ones may have gone unremoved in our wells, either because they were located deeper or because the fruitbodies collected in this study were totally decayed and locally dispersed by the microfauna at the sampling date.

Table 1 Unremoved fruitbodies on the six truffle grounds investigated (38 brûlés in all) after harvesting in 2018 or 2019, with extrapolation of the percentage (± standard deviation) of unremoved fruitbodies (see the "Materials and methods" section)

Tree species	Truffle harvest	Surface (m^2)	Well 1	Well 2	Well 3	Well 4	Unremoved (estimated, %)
Truffle ground #1 (2018)							
Q. ilex	11	19.6	1	0	0	0	83%
Q. ilex	37	28.3	5	0	0	0	91%
Q. ilex	6	12.6	0	0	0	0	0%
Q. ilex	7	6.2	0	0	0	0	0%
Q. ilex	50	20.4	0	1	0	1	69%
Mean of truffle ground #1 40 ± 33%							
Truffle ground #2 (2018)							
Q. ilex	19	3.1	4	0	0	0	65%
C. avellana	12	3.5	3	2	1	0	83%
C. avellana	11	18.1	0	0	0	0	0%
C. avellana	7	18.1	0	1	0	0	88%
Q. ilex	7	3.1	0	0	0	0	0%
Q. ilex	8	3.1	0	0	0	0	0%
Q. ilex	16	4.5	0	1	0	0	44%
Q. ilex	22	4.5	0	0	1	0	36%
Q. ilex	13	4.5	0	0	0	0	0%
Q. ilex	23	4.5	0	3	0	0	62%
Q. ilex	17	4.5	0	0	1	0	42%
C. avellana	14	6.2	0	1	0	0	55%
Mean of truffle ground #2 40 ± 33%							
Truffle ground #3 (2018)							
Q. ilex	4	13.8	0	0	0	2	95%
Q. pubescens	14	19.6	0	1	0	0	80%
Q. pubescens	8	19.6	1	0	0	0	87%
Q. pubescens	2	18.1	2	1	0	1	99%
Q. ilex	1	22.9	2	0	0	1	99%
Q. pubescens	1	13.8	0	0	0	1	97%
Q. ilex	4	21.2	2	0	0	6	99%
Q. pubescens	17	22.9	1	1	0	0	88%
Mean of truffle ground #3 93 ± 7%							
Truffle ground #4 (2019)							
Q. ilex	56	12.6	0	0	0	0	0%
Q. ilex	32	12.6	0	0	0	0	0%
Q. ilex	37	12.6	0	1	1	0	65%
Q. ilex	27	12.6	0	0	0	0	0%
Q. ilex	27	12.6	0	0	0	0	0%
Q. ilex	30	12.6	0	0	0	0	0%
Mean of truffle ground #4 11 ± 27%							
Truffle ground #5 (2019)							
Q. pubescens	14	4.5	1	0	1	0	64%
Q. pubescens	21	5.3	0	0	0	0	0%
Q. pubescens	17	5.0	0	0	0	0	0%
Q. pubescens	21	2.5	0	0	0	0	0%
Mean of truffle ground #5 16 ± 32%							
Truffle ground #6 (2019)							
Q. pubescens	29	10.2	0	0	0	0	0%
Q. ilex	17	10.2	0	0	0	0	0%

Table 1 (continued)

Tree species	Truffle harvest	Surface (m^2)	Well 1	Well 2	Well 3	Well 4	Unremoved (estimated, %)
Q. ilex	21	10.2	0	0	0	0	0%
Mean of truffle ground #6 0 ± 0%							
Mean of all truffle grounds 33 ± 33%							
Mean of all brûlés investigated 42 ± 41%							

The values revealed high variability among wells and among brûlés (Table 1), likely because of patchy distribution and low density of fruitbodies on the brûlé. The sampled area was very small (0.36 m^2/brûlé) and represented a small portion of the brûlés (from 1.3 to 14.2% of the brûlé surface, mean 4.9%); thus, the probability of detecting fruitbodies was low, even if they were present. In this framework, means are more relevant: by averaging values from the six truffle grounds, unremoved fruitbodies represented 33% of the whole fruitbody production; by averaging all the brûlés, this estimation reached 42% (Table 1). Due to their decaying status, no reliable weighting was possible, but unremoved truffles did not look smaller than fruitbodies harvested earlier in the season (Figure S1c).

Even for the mean calculated for the six truffle grounds, standard deviations were very high (as high as means; Table 1) because the diverse truffle grounds offer contrasting pictures. Unremoved fruitbodies reached 93% of the production in truffle ground #3, while none was found in truffle ground #6. We suspect that both local conditions and management practices, such as soil properties or the performance of dogs involved in harvesting, may explain these contrasting results. We tested whether truffle grounds on which many fruitbodies have been harvested during the season contain fewer unremoved truffles. However, no visual trend or statistically supported correlation was observed, either when comparing all brûlés (Figure S2A; linear regression model, $P = 0.17$) or when comparing this at truffle ground level (Figure S2B; linear regression model, $P = 0.46$). Thus, local conditions or practices (especially dogs) are unlikely to explain variable levels by a simple low-quality detection, and we really face a significant fraction of unremoved fruitbodies whatever the (removed) fruitbody production.

We hoped to convince more truffle growers to join our efforts in order to get a better evaluation, but our preliminary results met with some skepticism and limited the number of contributors. In the future, a larger set of truffle grounds will be useful to identify factors driving differences between them. To conclude, a substantial proportion of fruitbodies remain unremoved on average, even if this value is lower (and possibly null) in some truffle grounds.

Biological outcomes

Our results indicate that unremoved fruitbodies contribute to a truffle spore bank in truffle grounds. We cannot be sure that the germination ability of unremoved spores equals that of those deposited by feces (transit through the digestive tract may affect this parameter; Colgan and Claridge 2002). This inoculum may be subject to underground short-range dispersal by soil microfauna (e.g., truffle-eating coleopters such as *Leiodes cinnamomea* or larvae of the *Suillia pallida* fly; Le Tacon 2017). This may reinforce the IBD detected in *T. melanosporum* populations (Taschen et al. 2016; de la Varga et al. 2017) by allowing the settlement of genetically close individuals in the vicinity of their parents.

It is, however, rather unexpected to reach such a level of failed detection in truffle grounds where trained dogs pass regularly (more than once a week). It is hard to estimate the difference with wild truffle grounds (the high standard deviations precluded detection of statistical significance), but we suggest that the detection intensity may be at least similar in natural conditions. Indeed, if one assumes that unremoved fruitbodies contribute to IBD, the fact that IBD did not differ between managed and unmanaged (wild) truffle grounds (Taschen et al. 2016) suggests similar levels of failed detection.

Failed detection may be explained by two hypotheses (Fig. 1). First, accidentally, dogs may simply fail to detect some fruitbodies (as stated above, this is unlikely to be due to a deeper location): under this assumption, unremoved fruitbodies would be normal ones (Fig. 1, left panel). However, this scenario is unlikely when considering the frequent harvests on the truffle ground. Second, these fruitbodies may not be detectable by themselves, e.g., because they do not ripen correctly, remain odorless as they fail, during the ripening transition, to emit the aroma attracting dispersers (Fig. 1, right panel; Splivallo et al. 2011). At the time of detection in the wells, none of the fruitbodies had an aroma, but this was expected given their age and decay stage.

Absence of aroma emission, if any, may in turn result from two non-exclusive causes. First, developmental accident, unknown local conditions or parasitism may have modified fruitbody metabolism and aroma, but this is somewhat unexpected since, from our observations, the shape looked normal (Figure S1c). Second, it can be speculated that, during the development of fruitbodies, a developmental switch happens after which fruitbodies become either fragrant or not fragrant (Fig. 1, right panel). So far, we are only aware of intraspecific genotypic variability affecting aromas in the related species *T. aestivum* (Splivallo et al. 2012). Yet, a probability of not developing an attractive aroma may allow accumulation locally of spores that

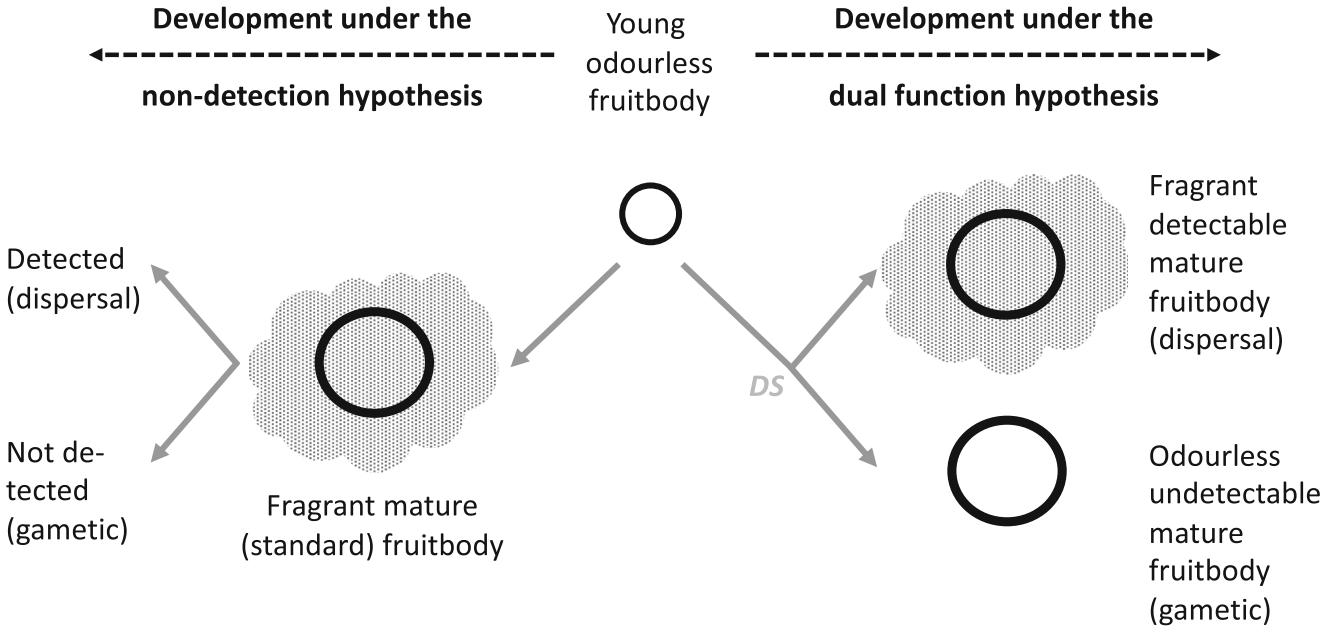

Fig. 1 Two non-exclusive hypotheses accounting for failed detection of *T. melanosporum* fruitbodies. Left, unremoved fruitbodies are identical (especially in aroma) to the removed ones, but were accidentally left over. Right, unremoved fruitbodies differ functionally from the other ones in a way precluding their detection: at a certain developmental stage (*DS*), some truffles may fail the shift to aroma production and thus remain undetectable

can contribute as male partners to mating in the years ahead. Thus, if spores are indeed recruited as a source of gametes, as is currently supposed (Selosse et al. 2013; Taschen et al. 2016; de la Varga et al. 2017), fruitbody development may have evolved to account for this; thanks to a developmental alternative. In other words, we suggest that developmental flexibility allows the production of undetectable fruitbodies (Fig. 1, right panel) in a process selected by way of paternal fitness. In more than 200 plant species, seeds undergo alternative development (Imbert 2002) resulting in heteromorphy associated with different functions, e.g., seeds with local versus distal dispersal ability, exactly as in our case for spores. However, the variable rates from one truffle ground to another suggest that such a mechanism, if any, is either spatially variable or does not account for the whole number of unremoved fruitbodies in some sites.

Implications for truffle production

The awareness that spores may provide a paternal contribution has prompted fears that production may be limited by the availability of paternal contributors, and this potentially gives meaning to two empirical practices (Taschen et al. 2016; Le Tacon 2017). First, the "truffle trap" is a hole in the brûlé refilled by a substrate containing *T. melanosporum* spores, which in some cases enhances production after 2 or 3 years (Murat et al. 2016; Taschen, Selosse & Richard, to be published). Second, many truffle growers carry out an annual scattering of fruitbody fragments on brûlés (Murat 2015) that are believed to sustain the presence of the fungus (re-inoculation). Indeed, it has been claimed that this practice explains why paternal diversity is higher in plantations where re-inoculation is performed than in the wild (spontaneous brûlés in the forest; Taschen et al. 2016). Our observations suggest that, if relevant from a sporal point of view, such practices may not be equally useful in every plantation, since variable amounts of spores are left in the soil. This calls for more careful evaluation of how truffle traps and annual scattering of fruitbody fragments contribute to production in diverse truffle grounds, and, as stated above, more studies on factors driving the amount of unremoved fruitbodies.

Finally, assessing why some fruitbodies are undetectable requires further analysis. If these fruitbodies are of commercial value at some point of their development, and as long as their partial harvest does not hamper the spore bank, there is a potential way of increasing (by up to one half) the production and income of truffle grounds.

Conclusion

We have quantified the proportion of unremoved *T. melanosporum* fruitbodies in managed truffle grounds where careful fruitbody harvesting makes leftovers improbable. The high proportion of leftovers contributes to the formation of a consequent and perennial spore bank as recorded for other hypogeous mushroom species and provides a potential source of male partners for mating in this species. We speculate that undetectability may even be selected for a fraction of fruitbodies and do not consider that the leftover would necessarily be of sufficient quality

for sale. The high variability of the percentage of unremoved fruitbodies from one truffle ground to another suggests that some as yet unknown factors affect it. Similar investigations for other truffle species, e.g., the economically important *T. aestivum* and *T. magnatum*, are now pending, although the absence of marked brûlé in these species may complicate delineation of the zone occupied by the mycelium.

Acknowledgments We are very grateful to Lucien Bonneau, Francis Caulet, Jean-Paul Laurents, Lucien Romieu, Patrick Savary, and Jean-François Tourette for having carried out the protocol on, or given access to, their truffle ground. We thank Dominique Barry-Etienne and Claude Murat for information on truffle fruitbody decomposition in soil, David Marsh for English corrections, two anonymous referees and Jan Colpaert for insightful comments on earlier versions of this paper, and Lucien Bonneau for providing pictures of the experiment (supplementary figure S1).

Authors' contributions LSM and MAS designed the study, contributed a new spore bank evaluation method, analyzed the data, and wrote the paper. All authors performed the research and improved the manuscript.

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