



Mitospore formation on pure cultures of *Tuber japonicum* (Tuberaceae, Pezizales) in vitro

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Abstract

The members of the genus *Tuber* are Ascomycota that form ectomycorrhizal associations with various coniferous and broadleaf tree species. In the teleomorphic stage, the species of the genus produce fruit bodies known as true truffles. Recent studies have discovered mitosporic structures, including spore mats, of several *Tuber* species on forest soils, indicating the presence of a cryptic anamorphic stage or an unknown reproductive strategy. Here, we report in vitro mitospore formation on the mycelium of *T. japonicum*, which belongs to the Japonicum clade, collected in several regions in Japan. Twenty of the 25 strains formed mitospores on modified Melin–Norkrans agar medium, indicating that mitospore formation is likely a common trait among strains of *T. japonicum*. The fungus forms repeatedly branched conidiophores on aerial hyphae on colonies and generates holoblastic mitospores sympodially on the terminal and near apical parts and/or occasionally on the middle and basal parts of the conidiogenous cells. Mitospores are hyaline and elliptical, obovate, oblong, or occasionally bacilliform, with a vacuole and often distinct hilar appendices. Formation of mitospores by *T. japonicum* in vitro is useful in understanding the functions of mitospores in the genus *Tuber* under controlled environmental conditions.

Keywords Anamorph · Conidia · Spore mat · Truffle

Introduction

The members of the genus *Tuber* (Tuberaceae, Pezizales, Ascomycota) are ectomycorrhizal (EcM) fungi that form symbiotic associations in the roots of various coniferous and broadleaf tree species (Bonito et al. 2013). The species of the genus produce underground tuberous fruiting bodies,

also known as “true truffles,” and the fruiting bodies of several European species, such as *T. magnatum* (Italian white truffle) and *T. melanosporum* (Périgord black truffle), have high market values as delicacies. Aiming for the improvement of truffle cultivation, massive studies are being conducted to understand the biology and ecology of the economically significant *Tuber* species (e.g., Marjanović et al. 2015; Todesco et al. 2019; Oliach et al. 2022).

Recent advances in molecular analysis unraveled the life cycles of several *Tuber* species, particularly *T. magnatum* (Paolocci et al. 2006), *T. melanosporum* (e.g., Martin et al. 2010; Rubini et al. 2011), and *T. borchii* (Belfiori et al. 2016). These studies revealed that *Tuber* species are heterothallic and predominantly haploid throughout their life cycle (Martin et al. 2010; Rubini et al. 2011). Meiotic ascospores produce haploid mycelia and each mycelial strain harbors one of two different mating genes (i.e., *Mat 1-1-1* or *Mat 1-2-1*). Sexual reproduction occurs between two strains with the opposite mating types. For *T. melanosporum*, it is proposed that one strain that form maternal tissue of ascocarps is prevalently present as ectomycorrhizas, while the other strain of the opposite mating type that involves mating with the strain is absent in ectomycorrhizas and

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may be present as germinating ascospores or habit in unknown ecological niches (e.g., Selosse et al. 2017). However, we still have a limited view of the process and mechanism of fertilization between strains of the two opposite mating types.

The role of putative asexual reproductive mode has recently gained significant attention as a hypothesis for the mechanism of fertilization in the life cycle of *Tuber*. The formation of either or both a meiospore (a haploid spore produced by meiosis) and mitospore (a haploid or diploid spore produced by mitosis) are reproductive modes found in several families of Pezizales (Paden 1972), such as Morchellaceae (Carris et al. 2015; Yuan et al. 2021). Mitospores are expected to contribute to dispersal as asexual spore-like conidia and/or reproduction as male gametes similar to spermatia. Field surveys in recent studies discovered spore mats of several *Tuber* species, including *T. borchii*, *T. oligospermum* (Puberulum clade; Urban et al. 2004), three undescribed species belonging to Maculatum and Puberulum clades (Healy et al. 2012), and *T. brennemanii* (Maculatum clade; Grupe et al. 2018), as well as the other EcM fungi of Ascomycota on forest grounds, such as the genus *Hydnobolites* and *Pachyphlodes* (Healy et al. 2012). So far, mitospore formation have not been reported in other members of the genus *Tuber*. These studies indicated that the asexual reproductive mode of mitospore formation is present, at least within the two clades, in the genus *Tuber*. However, no study has yet demonstrated the induction of mitospore formation from the mycelium of *Tuber* strains in vitro.

Tuber japonicum Hir. Sasaki, A. Kinosh. & Nara is endemic to Japan and is widely distributed in Honsyu Island (Kinoshita et al. 2011, 2016). Ascocarps of the fungus are relatively large (4 cm in diameter or larger), whitish with a distinctive aroma (Shimokawa et al. 2020), and grow in acidic soil (pH 5–6) with poor soil nutrient status (Furusawa et al. 2020) under both coniferous (*Abies* and *Pinus*) and broadleaf trees (*Betula*, *Carpinus*, *Lithocarpus*, and *Quercus*) in woodlands. Thus, several studies for the artificial cultivation of the truffle species are now ongoing in Japan (Kinoshita et al. 2018; Nakamura et al. 2020; Nakano et al. 2020, 2022). We have never found spore mats at the sites where ascocarps of *T. japonicum* occurred, but discovered mitospore formations on mycelia of several pure cultures of the species. This study aimed to examine if mitospore formation is a common trait among *T. japonicum* strains obtained from different regions in Japan and to describe the morphological characteristics of the mitosporic structures.

Materials and methods

Fungal materials

Twenty-five strains of *T. japonicum* were used in this study (Table 1). They were isolated from gleba using the method described by Nakano et al. (2020). The strains were

deposited in the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan. Each strain was maintained on a modified Melin–Norkrans (MMN) agar medium (Marx 1969) or yeast-glucose agar medium (Tanaka and Nara 2009) in a Petri dish (9 cm in diameter) in the dark at 23–25 °C until use.

Microscopic observation of mitosporic structures

Mitosporic structures were discovered by coincidence on colonies of four *T. japonicum* strains: (1) on the colony of the FFPRI 460538 strain, which was maintained by subculturing on MMN agar medium, (2) on the colony of the FFPRI 460544 strain, which was maintained for 6 months on MMN agar medium after isolation from gleba, and (3) mycelia of the FFPRI 460542 and FFPRI 460545 strains, which were incubated for three months in modified MMN liquid medium with 500 mg of potassium nitrate instead of 250 mg of di-ammonium hydrogen phosphate and 1.0 g instead of 3.0 g of malt extract added for 1-L media.

The mitosporic structures were observed under a light microscope (BX53, OLYMPUS, Tokyo, Japan) and a cryo-scanning electron microscope (cryo-SEM) (JSM6510A; JEOL, Tokyo, Japan). For the light microscope, the mycelia or mycelial agar blocks, including mitospores of each *T. japonicum* strain on the MMN medium, were cut using a micro spatula, placed on a glass slide, and mounted with distilled water. Light micrographs were taken using a digital camera under 40–100× magnification (D5300, Nikon, Tokyo, Japan).

For the cryo-SEM, the mycelial agar blocks of the FFPRI 460542 strain was attached to a specimen holder using the frozen embedding medium (Shandon Cryomatrix, Thermofisher Scientific, Tokyo, Japan), which was cryofixed by direct immersion in cooled hydrochlorofluorocarbon at –150 °C and transferred to the cold stage of a cryo-SEM system. Secondary electron images were obtained at an acceleration voltage of 3 kV at –160 °C (Kuroda et al. 2003, 2018).

Examination of mitospore formation frequency and mitospore size

We examined mitospore formation on pure cultures of 17 *T. japonicum* strains obtained from different regions in Japan to confirm if the ability of mitospore formation is a common trait among the strains and retained during subculturing under the same storage conditions. Most strains had been maintained by subculturing for more than 2 years after isolation before the experiments. Each strain was incubated on 20-mL MMN agar medium in a petri dish (9 cm in diameter) for 1 month in the dark at 23–25 °C, and then, mycelial disks containing growing mycelia were extracted from the

Table 1 Culture strains of *Tuber japonicum* used in this study and characteristics of mitospores

Strain	Collection date	Collection site	Putative host tree ^a	Accession no. (ITS)	Mitospore formation ^b	Frequency (f) ^c			Size of mitospores		Q ^d	n
						First	Second	Third	Size (µm)	Q ^d		
003804	2015	Hirakata, Osaka	Unknown	LC672617	○	1	0	0	n.d.	n.d.	n.d.	n.d.
FFPRI 460518	Dec, 2016	Hirakata, Osaka	Qs	LC388742	○*	2	2	3	3.3–5.3×6.3–9.2 (–10.7)	1.3–2.6 (–2.9)	32	
FFPRI 460536	Dec, 2016	Hirakata, Osaka	Qs	LC672618	×	0	0	0	-	-	-	-
FFPRI 460515	Nov, 2016	Inabe, Mie	Pd, Qs	LC388741	○	3	3	3	n.d.	n.d.	n.d.	n.d.
FFPRI 460542	Nov, 2018	Inabe, Mie	Pd, Qs	LC672619	○*	0	0	0	2.9–5.2 (–5.4)×5.9–10.3 (–10.4)	1.4–2.9 (–3.2)	50	
FFPRI 460543	Nov, 2018	Inabe, Mie	Pd, Qs	LC672620	○*	1	1	1	3.1–5.1 (–5.3)×5.3–9.4 (–11.0)	1.2–2.6 (–2.7)	50	
FFPRI 460544	Nov, 2018	Inabe, Mie	Pd, Qs	LC672621	○	1	1	2	3.3–5.1×5.0–9.6 (–12.5)	1.1–2.7 (–3.0)	50	
FFPRI 460545	Nov, 2018	Inabe, Mie	Pd, Qs	LC672622	○*	0	0	3	2.7–5.1×6.0–12.0 (–12.3)	1.3–3.9	50	
FFPRI 460516	Nov, 2016	Inabe, Mie	Cc, Pd, Qs	LC430613	○	0	3	0	n.d.	n.d.	n.d.	n.d.
FFPRI 460517	Nov, 2016	Inabe, Mie	Cc, Pd, Qs	LC430614	○*	1	0	1	(2.7–)2.9–4.7×5.8–10.6 (–11.5)	1.4–3.1 (–3.3)	50	
FFPRI 460541	Dec, 2016	Kobe, Hyogo	Qd	LC672623	×	0	0	0	-	-	-	-
003805	2015	Sanda, Hyogo	Qa, Qs	LC672624	×	0	0	0	-	-	-	-
003806	2015	Sanda, Hyogo	Qa, Qs	LC672625	○*	0	0	2	n.d.	n.d.	n.d.	n.d.
S75-1	Oct, 2019	Nogi, Tochigi	Csp, Qs	LC672626	○*	3	3	3	3.8–5.9×7.8–16.4 (–20.8)	1.4–3.9 (–4.5)	50	
003803	Dec, 2014	Omitama, Ibaraki	Qs	LC672627	×	0	0	0	-	-	-	-
003802	Dec, 2014	Omitama, Ibaraki	Qs	LC672628	○*	0	3	3	3.6–5.7×6.7–12.8 (–13.6)	1.4–3.1 (–3.2)	50	
FFPRI 460514	Dec, 2015	Wake, Okayama	Cs, Qm	LC430612	×	0	0	0	-	-	-	-
FFPRI 460538	Dec, 2016	Sanda, Hyogo	Unknown	n.d.	○	n.d.	n.d.	n.d.	2.8–5.3×5.0–10.6	1.2–2.9 (–3.5)	50	
OBASE00203	Nov, 2020	Omitama, Ibaraki	Qa, Qs	LC672629	○*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
OBASE00205	Nov, 2020	Inabe, Mie	Qsp	LC672630	○	n.d.	n.d.	n.d.	3.3–4.9×5.3–8.6 (–10.0)	1.2–2.6 (–2.7)	50	
OBASE00207	Nov, 2020	Inabe, Mie	Qs	LC672631	○*	n.d.	n.d.	n.d.	3.1–4.8×4.7–8.9	1.1–2.4	33	
OBASE00208	Nov, 2020	Kobe, Hyogo	Qd	LC672632	○*	n.d.	n.d.	n.d.	3.4–5.2×5.6–9.0 (–10.1)	1.1–2.3 (–2.4)	50	
OBASE00209	Nov, 2020	Nogi, Tochigi	Csp, Qs	LC672633	○*	n.d.	n.d.	n.d.	3.5–5.3 (–5.4)×5.7–9.2 (–12.0)	1.2–2.6 (–3.3)	50	
OBASE00210	Nov, 2020	Hirakata, Osaka	Qs	LC672634	○*	n.d.	n.d.	n.d.	3.5–5.2×5.8–12.1	1.2–2.8 (–3.2)	50	
OBASE00213	Nov, 2020	Inabe, Mie	Pd	LC672635	○*	n.d.	n.d.	n.d.	3.2–5.6×6.1–9.8 (–10.3)	1.2–2.5 (–2.9)	50	

n.d., not determined

^aMain tree species in the area where the ascocarps were collected: Cc, *Castanopsis cuspidata*. Cs, *Castanopsis sieboldii*. Csp, *Carpinus* sp. Pd, *Pinus densiflora*. Qa, *Quercus acutissima*. Qd, *Quercus dentata*. Qm, *Quercus myrsinifolia*. Qs, *Quercus serrata*. Qsp, *Quercus* sp

^b○, mitospore formation have been found. ○*, ITS sequences derived from mycelia with mitospores were confirmed to be identical with those of mycelia. ×, mitospore formation was never found

^cThe number of culture plates in which mitospores were formed during three successive subculturing

^dRatio of length to width

cultures using an 8-mm cork borer. The disks were placed individually in the center of a 20-mL MMN medium and incubated in the dark at 23–25 °C. The experiment was conducted three times (in June 2020, September 2020, and June 2021), with three replications for each strain each time. The surfaces of 30–45-day-old colonies were observed under a light microscope (BX60, OLYMPUS, Tokyo, Japan).

To examine mitospore formation on relatively young strains, i.e., not preserved for a long time and have not yet undergone successive subculturing, colony surfaces of additional seven strains (OBASE00203–213), which were isolated in November 2020 and subcultured three times at 3-month intervals, were observed under a light microscope.

To examine the size of mitospores, mitosporic structures on the preserved cultures of each strain were cut with a scalpel and mounted with distilled water. Light micrographs were taken under a light microscope (BX53, OLYMPUS, Tokyo, Japan) using a digital camera at 100× magnification (D5300, Nikon, Tokyo, Japan), and the size of the mitospores was measured using PhotoRuler 1.1 (http://www.inocybe.info/_userdata/ruler/PhotoRuler.html).

Molecular confirmation

Mitospores were collected from 2-month-old colonies of *T. japonicum* strain S75-1 incubated on 20 mL of MMN agar medium in ten 50-mL conical tubes (352098, Falcon, Mexico) by dripping 2 mL of 70% ethanol aqueous solution onto the colonies, increasing the hydrophilicity of the mitospores, and making them easier to collect into the solution, and decanting it into other conical tubes.

The collected solution was filtered through a 15-µm membrane filter (43-50015-01, Pluristrainer). Mitospores were successfully recovered using this method, although a small amount of mycelium fragments were also mixed with the mitospores. Since the mitospores likely account for a high percentage of the total DNA obtained from this sample, we judged that clear sequences obtained from these samples were derived from mitospores. When a clear sequence of *T. japonicum* was not obtained from the sample, it likely means that mitospores were derived from contaminating fungi. Collected solutions were dispensed into multiple 1.5-mL micro-tubes and centrifuged at 14000 rpm for 5 min. The supernatant was removed by pipetting and evaporating at room temperature. Fungal DNA was extracted as described by Izumitsu et al. (2012).

The internal transcribed spacer (ITS) region of ribosomal RNA gene was amplified under nested polymerase chain reaction (PCR) using Tks Gflex DNA Polymerase (Takara, Otsu, Japan) with primer pairs NSA3 and NLC2 (Martin and Rygielwicz 2005) for the first PCR and ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for the second PCR because the amount of DNA was considered to be low.

PCR conditions were as follows: 5 min at 95 °C; 35 cycles of 20 s at 95 °C, 20 s at 55 °C and 30 s at 68 °C; and 5 min at 68 °C. The methods of purification and Sanger sequencing were the same as described by Nakamura et al. (2020). We judged that fungal contaminants were absent in the samples if clear sequences identical to those of *T. japonicum* were obtained, and mitospores were derived from pure strains of *T. japonicum*.

We also extracted DNA from mycelia with massive mitospores on the culture plate of several strains and sequenced the ITS region using the method described by Obase et al. (2021) to see if clear sequences, which are identical to those of *T. japonicum*, were obtained from the samples.

Statistical analysis

We conducted Fisher's exact test using PAST v.3.01 to examine if the ratio of the number of cultures, which induced mitospore formation, differed between relatively young (OBASE00203-213) and old strains (Hammer et al. 2001).

Results

Confirmation of mitospore formation on nutrient medium

Mitospore formation was induced in 20 of 25 strains of *T. japonicum* on MMN agar or liquid medium (Table 1). Clear ITS sequences were successfully obtained from mitospores of the strain S75-1 and mycelia with mitospores of 13 strains tested. All sequences were identical to those of *T. japonicum* mycelial strains.

Of 17 strains tested, five strains (FFPRI 460515, FFPRI 460518, FFPRI 460543, FFPRI 460544, and S75-1) formed mitospores in all three successive experiments (Table 1). Mitospores were not found in any of the three successive experiments in the other five strains (003804, 003806, FFPRI 460516, FFPRI 460517, and FFPRI 460545). Seven strains, including the FFPRI 460542 strain, which was the first strain in which mitospore formation was observed on its colony, never formed mitospores during the experiments. All seven young strains (OBASE00203-213) were isolated in November 2020 and subjected to three successive subculturing, resulting in the formation of mitospores.

Morphological characteristics of cultures and mitospores in *T. japonicum*

In this study, we conveniently applied the terminology used to describe conidial structures and conidiogenesis. Colonies are whitish to yellowish, filamentous, and flat or raised

(Fig. 1a, b). No exudates or soluble pigments were observed. Basal hyphae are 3.7–11.5 μm wide, hyaline, smooth, and septate. Hyphal aggregates (Iotti et al. 2002; Obase et al. 2021) and vesicle-like structures are found in several strains. Anastomoses are commonly found. Colony diameter after 1 month incubation varied significantly among strains, ranging from 1.2 to 7.0 cm.

Conidiophores are whitish, solitary, or gregarious on colonies (Fig. 1a, b, d) and develop laterally or terminally on undifferentiated aerial hypha formed by septate, hyaline, and repeatedly branched hyphae (Fig. 1c). The structures were formed around the inoculum or unspecified locations within colonies, generally 30–45 days, but rarely within 1 week (strain S75-1) after incubation. Conidiogenous cells are hyphal, septate, and generally originate from the apical parts of the terminal cells of conidiophore branches, but the boundaries are occasionally ambiguous because mitospore formation is seen on the middle parts of the conidiophore branches and is not limited to the terminal cells in the FFPRI 460542 strain (Fig. 1e, g, h). Conidiogenous cells extend and often branch 2–3(–4) in verticils in a 45° direction from the axis (Fig. 1c). Conidiogenesis is holoblastic (Fig. 1e), sympodulosporous, forming one to several mitospores densely on the apical parts or at the side parts of conidiogenous cells directly above the septa (Fig. 1c, e). Conidial scars were inconspicuous.

Mitospores are smooth, thin-walled, aseptate, and colorless, with a vacuole and distinct hilar appendices (Fig. 1f). Shapes of the mitospores are rarely subglobose, often elliptical and obovate to oblong; however, several strains also contained many bacilliform or allantoid mitospores. The average size of mitospores among strains is 4.3 \times 8.5 μm , with a basal hilus (Table 1). The average ratio of length to width (Q) is 2.0. Mitospores and conidiophores are hydrophobic.

Discussion

This is the first study to demonstrate in vitro mitospore formation induced by pure cultures of a *Tuber* species. It is also the first study to report mitospore formation in *Tuber* species of the Japonicum clade. So far, several species of the Puberulum and Maculatum clades have been identified as spore mat-forming species in situ (Urban et al. 2004; Healy et al. 2012; Grupe et al. 2018). Although spore mats of *T. japonicum* have not been reported in the field, and it is uncertain if *T. japonicum* formed mitospores under field conditions, the findings of this study support the idea that the taxa with the anamorphic mode are likely more prevalently distributed in the phylogeny of the genus *Tuber* than previously thought because the Japonicum clade is thought to be one of the early diverging lineages in the genus *Tuber* (Bonito et al. 2013), and thus, it is possible that the ability

of mitospore formation is an ancestral character shared by other members of the genus.

Surprisingly, the cultures of *T. japonicum* formed mitospores on MMN agar medium, which is commonly used for culturing EcM fungi without any special treatments, i.e., manipulating environmental conditions (e.g., nutrient status of the media, humidity, and temperature), even though we have never succeeded in mitospore formation in pure cultures of other *Tuber* species, including several species of the Puberulum and Maculatum clades collected in Japan (Obase et al. 2021) under the same condition of the current study. Some *Tuber* species might be able to form mitospores whereas others are unable to, or the preferable conditions for mitospore formation differ among *Tuber* species. Nutrient condition on MMN media with stable environmental conditions was rather acceptable for mitospore formation for several *T. japonicum* strains.

Twelve of the 17 strains formed mitospores on MMN agar medium; however, the remaining five strains never formed them during the experiments. The results indicate a variation in ability or preferable conditions for mitospore formation among strains of *T. japonicum*. Whether or not the nutritional composition of the nutrient media in which the strains grow, as well as environmental conditions, such as moisture and temperature, affect mitospore formation remains to be examined. However, there may be an effect of either or both mutation and degeneration of the strains during successive subculturing, which can result in the loss of conidium formation ability (e.g., Shah et al. 2007). Although there was no significant difference in the frequency of strains, which induced mitospore formation between relatively young (7/7; the number of strains that induced mitospore formation/total numbers of strains) and old strains (13/18) ($p=0.27$, Fisher's exact test), further studies are required to determine the effects of successive subculturing on the ability to form mitospores in *Tuber* strains.

The morphology of conidiophores and pattern of mitospore development in *T. japonicum* had similar characteristics with those of other *Tuber* species, i.e., conidiogenesis is holoblastic and sympodulosporous. However, there are distinct differences in the parts of mitospore formation and the structure of the mitospores. Previous studies discovered mitospores on the apical parts of conidiogenous cells in *T. borchii* and *T. oligospermum* (Urban et al. 2004), but mitospores were formed not only on apical parts but also at the side parts of conidiogenous cells directly above the septa in all *T. japonicum* strains. The FFPRI 460542 strain, which was incubated in MMN liquid medium, also occasionally formed mitospores on the middle parts of the conidiophore branches and primarily on terminal cells. Next, *T. japonicum* forms mitospores that are longer (8.5 μm on average, up to 20.8 μm) than those of other *Tuber* species: around 5 μm long in *T. borchii* (Urban et al. 2004) and 3–6 μm long in *T.*

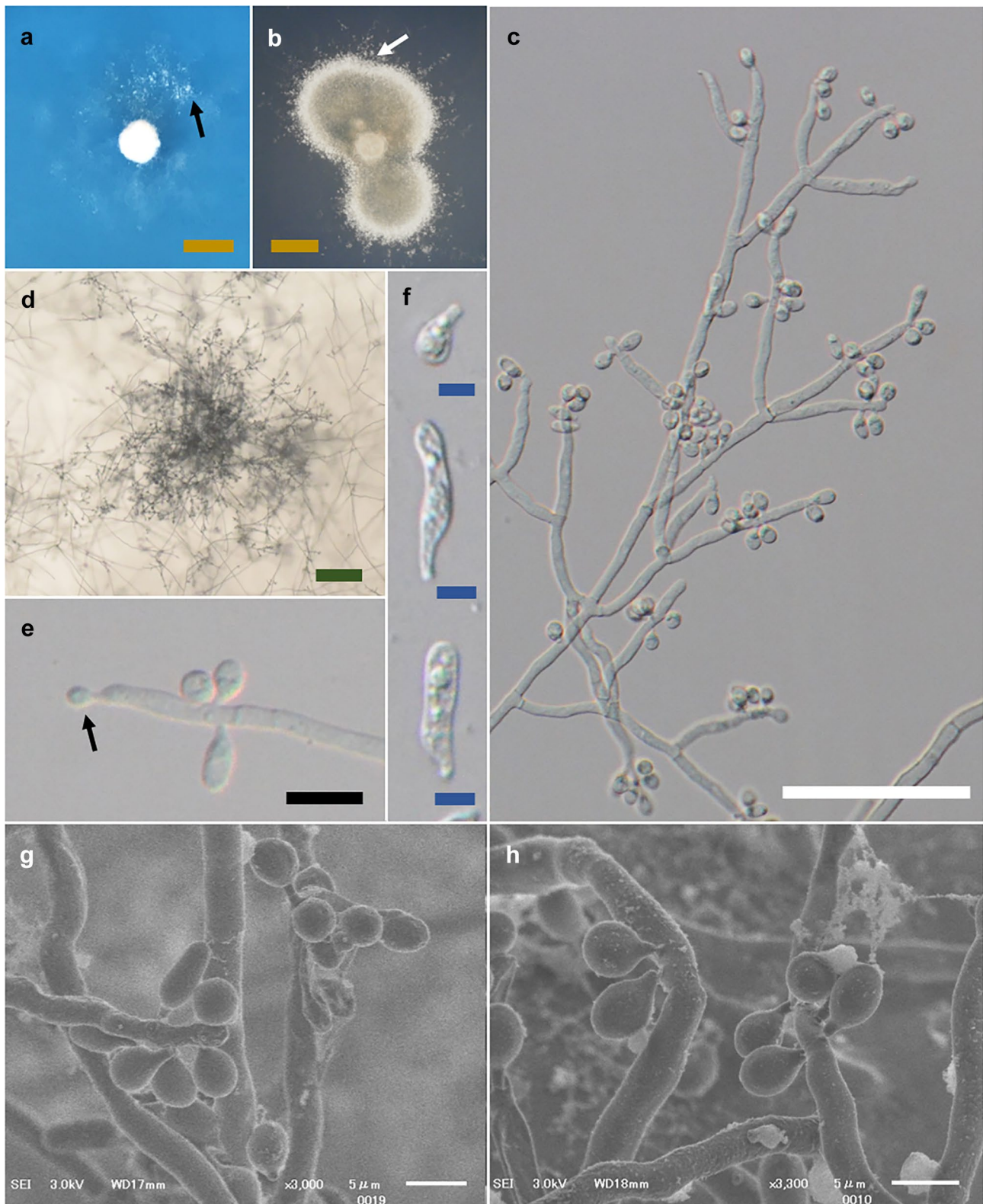


Fig. 1 Mitospore formation on pure cultures of *Tuber japonicum* on MMN agar medium. **a, b** Mycelial colonies of FFPRI 460542 (**a**) and FFPRI 460543 (**b**) strains. Arrows indicate conidiophores. **c** Structures of conidiophores in the strain FFPRI 460542. **d** Conidiophores on the colony of the FFPRI 460516 strain. **e** Holoblastic conidiogen-

esis in the strain FFPRI 460542. **f** Obovate and cylindrical to bacilliform mitospores in the S75-1 strain. **g, h** Cryo-scanning electron micrographs for conidiogenesis in the strain FFPRI 460542. Bars indicate 1 cm (**a, b**), 50 μm (**c**), 100 μm (**d**), 10 μm (**e**), and 5 μm (**f–h**), respectively

brennemanii (Grupe et al. 2018). Although comparing the shape of mitospores formed in vitro with those formed on spore mats in the field may not be reasonable, the results suggest that there might be a difference in the pattern of mitospore formation and morphology of mitospores among *Tuber* species.

So far, only a single study has attempted to understand the functions of mitospores of *Tuber* species. Healy et al. (2012) failed to germinate mitospores of *Tuber* spp. collected from the field on axenic nutrient media and was unable to form ectomycorrhiza by inoculating the mitospores on seedlings of several tree species, concluding that mitospores may act as male gametes rather than as asexual spores. However, because the quality of mitospores (e.g., viability and contamination by other microbes) obtained from the field is uncertain and the environmental conditions for the mitospores to function (e.g., temperature, humidity, and nutrition, and duration needed for germination) are unknown, additional operational tests with high-quality and massive mitospores are necessary to understand the function of the mitospores. *Tuber japonicum*, which can meet the above requirements by inducing mitospore formation in vitro using pure cultures, could be used as a model species to understand the functions of the mitospores in *Tuber* spp. in the future.

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Declarations

All experiments conducted in this study comply with the current Japanese laws.

Conflict of interest The authors declare no competing interests.

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