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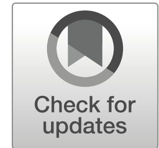

Repeated fruiting of Japanese golden chanterelle in pot culture with host seedlings

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Abstract

Yellow chanterelles are among the most popular wild edible ectomycorrhizal mushrooms worldwide. The representative European golden chanterelle, *Cantharellus cibarius*, has only once been reported to fruit under greenhouse conditions, due to the difficulty of establishing pure culture. Recently, we developed a new technique for establishing a pure culture of a Japanese golden chanterelle (*Cantharellus anzutake*), and conducted in vitro ectomycorrhizal synthesis using established strains and *Pinus densiflora*. Acclimated pine mycorrhizal seedlings colonized with *C. anzutake* in a pot system under laboratory conditions produced small but distinct basidiomata with developed basidiospores. *C. anzutake* mycorrhizae were established on *Quercus serrata* seedlings by inoculation of mycorrhizal root tips of the fungus synthesized on *P. densiflora*. A scaled-up *C. anzutake*–host system in larger pots (4 L soil volume) exhibited repeated fruiting at 20–24 °C under continuous light illumination at 150 μmol m^{-2} s^{-1} during a 2-year incubation period. Therefore, a *C. anzutake* cultivation trial is practical under controlled environmental conditions.

Keywords *Cantharellus* · Edible ectomycorrhizal mushroom · Cultivation · Symbiosis

Introduction

Yellow chanterelles (*Cantharellus* spp., order Cantharellales; Hibbett et al. 2014) have long been among the most popular groups of wild edible ectomycorrhizal mushrooms in Europe and North America, and recently in Africa and Asia as well (Corner 1966; Persson 1997; Watling 1997; Pilz et al. 2003, 2006; Arora and Dunham 2008; Buyck et al. 2014). The market demand for this mushroom group has motivated global-scale studies of its taxonomy and biodiversity (e.g., Buyck 2016). At present, over 100 chanterelle species have been described, some of which are harvested as edible mushrooms. Popular species found in markets include *Cantharellus cibarius* Fr. and *C. pallens* Pilát in Europe, and *C. formosus* Corner and *C. roseocanus* (Redhead, Norvell & Danell) Redhead, Norvell & Moncalvo in North America (Pilz et al. 2003). The yellowish color characteristic of many chanterelles and their unique, apricot-like fragrance contribute to the attraction of these mushrooms.

Yellow chanterelles lack true gills, but develop gill-like ridged hymenia on the basidioma (Buyck et al. 2014; Olariaga et al. 2016). The unique phylogenetic position of

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Cantharellus in the agaric clade of Basidiomycota confers specific biological characteristics, e.g., the presence of endofungal microorganisms including bacteria and fungi in basidioma tissue (Danell et al. 1993; Danell 1999; Kumari et al. 2013). These microorganisms make it difficult to establish pure chanterelle cultures from their basidiomata tissues. Therefore, cultivation studies of this mushroom group are quite limited, and few cultured strains are available. However, spore isolation from *C. cibarius*, the genus type species, is applicable when optimal medium conditions are prepared for germination (Fries 1979; Danell and Fries 1990).

The first and only successful cultivation study of chanterelles was conducted approximately two decades ago. Danell and Camacho (1997) reported *C. cibarius* fruiting under greenhouse conditions, in which inoculated *Pinus sylvestris* seedlings were acclimated in vitro with *C. cibarius* in open pots. Subsequently, the fruiting of this fungus was repeatedly observed over a period of several months (Pilz et al. 2003). However, this trial did not lead to the development of a field cultivation/mushroom harvest system in situ. As this fruiting event was observed repeatedly and the technique was based on an elegant and reliable in vitro ectomycorrhization procedure (Danell 1994), reproducing this fruiting trial has been an important research goal. Chanterelle mycorrhization in vitro has been achieved by other researchers through the use of pure culture strains and axenic plants (Sharma et al. 2008, 2011). However, it remains unclear whether these cultures were truly chanterelles, due to their quite different mycelial growth characteristics (Sharma et al. 2010) compared with those of *C. cibarius* and *C. anzutake* (Straastma et al. 1985; Pilz et al. 2003; Ogawa et al. 2019), and a lack of phylogenetic data supporting their taxonomic identity.

We recently described the Japanese golden chanterelle *C. anzutake*, a sensu *lato* cryptic species of *C. cibarius* from Japan (Ogawa et al. 2018), and established its pure culture from ectomycorrhizal root tips (Ogawa et al. 2019). This fungus was formerly identified as *C. cibarius* Fr. in Japan and is known among local people as an edible mushroom (Kawamura 1908, 1955; Imazeki and Hongo 1989). *C. anzutake* and *C. cibarius* are very similar in morphology and phylogenetic position, suggesting similar market value. However, *C. anzutake* differs from *C. cibarius* in its geographic distribution and habitat, i.e., cool to warm temperate regions in Far East Asia, where it is likely endemic. Culture characteristics are also similar between the two species: cream to yellowish colonies, fragrant apricot fruit, and clamp connections (Straastma et al. 1985; Pilz et al. 2003; Ogawa et al. 2019). As the full genome sequence of one of our established *C. anzutake* cultured strains (C-23) has already been uploaded to the Joint Genome Institute (JGI) genome portal (https://genome.jgi.doe.gov/portal/), DNA-based biological characterization is now possible for this fungus. Therefore, studies of *C. anzutake* cultured strains will elucidate their physiology and genetics, as well as the mechanisms of fungus–plant interaction in yellow chanterelles.

The objectives of this study were to establish ectomycorrhizae of *C. anzutake* in vitro and to induce mushroom fruiting using acclimated plants. We also aimed to repeatedly induce *C. anzutake* basidiomata under controlled environmental conditions.

Materials and methods

Fungal cultures

We used seven cultured strains of *C. anzutake* that were established in 2009–2011 (Table 1; Ogawa et al. 2019). Of these strains, five (EN-51, EN-52, EN-53, EN-60, and EN-61) were tested for in vitro ectomycorrhizal synthesis to examine their colonization on pine seedlings and to acclimate the ectomycorrhizae obtained under non-sterile conditions. Because two of the five tested strains fruited in a 250-mL jar experiment (see below), another two strains (EN-98 and C-2) were similarly tested for in vitro ectomycorrhizal synthesis on

Table 1 Cultured strains of *Cantharellus anzutake*

Strain ID	Isolation origin				Culture deposit on NBRC[1]
	Year	Fungal structure	Forest canopy species	Sampling site in Japan	
EN-51	2009	Basidioma	*Quercus acutissima*, *Q. serrata*	Nakagawa, Nagano Prefecture	113266
EN-52	2010	Basidioma	*Picea abies*	Minami-minowa, Nagano Prefecture	113267
EN-53	2010	Basidioma	*P. abies*, *Betula platyphylla var. japonica*, *Q. serrata*	Minami-minowa, Nagano Prefecture	113268
EN-60	2010	Ectomycorrhizae	*P. abies*	Minami-minowa, Nagano Prefecture	113269
EN-61	2010	Ectomycorrhizae	*P. abies*	Minami-minowa, Nagano Prefecture	113270
C-2	2011	Ectomycorrhizae	*Pinus luchuensis*	Kumejima Island, Okinawa Prefecture	
EN-98	2011	Ectomycorrhizae	*Q. serrata*, *Pinus densiflora*	Nakagawa, Nagano Prefecture	

[1] NBRC: NITE Biological Resource Center, National Institute of Technology and Evaluation (NITE; http://www.nite.go.jp/en/index.html)

pine seedlings. The mycorrhizal seedlings obtained were used for subsequent scale-up experiments.

Ectomycorrhiza synthesis on a pine host

We tested in vitro ectomycorrhization using the five *C. anzutake* strains mentioned above and *P. densiflora* as the host, based on previously reported ecological data for this fungus (Ogawa et al. 2018, 2019). The growth of stored slant cultures of these strains was recovered on modified Norkrans C (MNC) medium agar plates (Yamada and Katsuya 1995). A 1 × 1-cm mycelial culture excised from a colony on an MNC agar plate was inoculated into 10 mL MNC liquid medium in a 75-mL wide-mouth glass bottle (5-128-01, UM sample bottle; AS ONE Corp., Osaka, Japan) and was grown at 20 °C for 30 days prior to inoculation into the host. Twenty bottles per *C. anzutake* strain were prepared. Pine seeds gifted from the Nagano Prefectural Forestry Center and stored at 5 °C in a refrigerator were axenically germinated on MNC agar plates (Endo et al. 2014), and 5- to 10-day-old seedlings were used for ectomycorrhiza synthesis.

For each sample, a 150-mL wide-mouth glass bottle (5-128-02, UM sample bottle; AS ONE Corp.) was filled halfway with a vermiculite/sphagnum moss mixture (40:1, *v*/*v*) saturated with MNC liquid medium with reduced glucose concentration (2 g/L). Approximately 200 mL MNC liquid medium was required to saturate the 500-mL vermiculite/sphagnum moss mixture. This bottle was closed with a vented (diameter, 5 mm) polycarbonate cap, and the vent was sealed with Milliseal (Merck KGaA, Darmstadt). These prepared 150-mL glass bottles were autoclaved at 121 °C for 20 min and cooled on a clean bench for further manipulation. A mycelium (~ 0.2 g fresh weight) grown in a 75-mL glass bottle was dissected into several segments with fine forceps and inoculated to the vermiculite/sphagnum moss mixture at the middle depth in a prepared 150-mL glass bottle, and a germinated pine seedling was then transplanted into the inoculated bottle. Twenty replicates were set for each of the five tested *C. anzutake* strains. The co-culture bottles were incubated in an illuminated incubator (FLI-2000A, TOKYO RIKAKIKAI Co., Ltd., Tokyo) at 20 °C under continuous illumination by fluorescent light at 140 μmol m^{-2} s^{-1} for 6–10 months. Autoclaved distilled water (~ 20–30 mL per bottle) was added axenically every 2 months for each seedling.

After the ectomycorrhiza synthesis, all pine seedlings were removed from the glass bottles, and the root systems were washed under flowing tap water and observed under a stereomicroscope (Stemi 2000-C, Carl Zeiss, Jena). Approximately 1/8 of the long roots was removed from the entire root system for the counting of fine and ectomycorrhizal root tips to estimate ectomycorrhizal colonization ratio per seedling. Several ectomycorrhizal root tips were then prepared for further microscopy.

Acclimation of in vitro synthesized ectomycorrhizal seedlings under non-sterile conditions

We tested whether *C. anzutake* ectomycorrhizae synthesized in vitro could grow under non-sterile conditions in organic and mineral soils. Mineral soil was sampled from a pine forest site established on granitic parent rock in the Tera Experimental Forest (35° 53′ 39″ N, 138° 2′ 29″ E), Faculty of Agriculture, Shinshu University. Organic soil was sampled from a pine forest site established on volcanic andosol soil on the campus of the Faculty of Agriculture, Shinshu University (35° 52′ 4″ N, 137° 56′ 4″ E). Both soil samples were collected from the forest floor with a shovel, sieved through standard 1-cm mesh, dried at 50 °C, and stored in the laboratory until use.

The sampled soils were sieved (mesh size, 5 mm) and autoclaved at 121 °C for 60 min. A 200-mL autoclaved soil sample was placed in a 250-mL wide-mouth polystyrene jar (PS-250, AS ONE Corp.), and an ectomycorrhizal pine seedling, the root system of which had been washed gently with flowing tap water to remove vermiculite and sphagnum moss particles, was transplanted into the jar. This plant jar was capped with another inverted jar, and the joint was sealed with polyvinylchloride film (Riken tape, RIKEN TECHNOS GROUP, Tokyo). The top jar had two vents (diameter, 5 mm) that were sealed with Milliseal. In total, 79 jars (12–18 jars for each of five fungal strains tested) were set in a biotron at 23 °C under continuous illumination by fluorescent light at 100 μmol m^{-2} s^{-1}, and incubated for ~ 10 months. The bottom jar was covered with aluminum foil to mask it from light. Irrigation was performed monthly and alternately with distilled water (30–50 mL) and the same volume of a solution of Hyponex 6–10–5 (HYPONeX Japan Co., Ltd., Osaka) diluted to a ratio of 1:2,000 with distilled water. If *C. anzutake* fruiting structures occurred, they were observed both macroscopically and microscopically, with particular attention to the formation of hymenium and basidiospores.

Two *C. anzutake* strains (EN-98 and C-2) were later used for in vitro ectomycorrhizal synthesis with *P. densiflora* after EN-61 basidioma formation (see below) because they had different isolation origins (Table 1) and were expected to have different physiological properties. We used four and 10 pine seedlings for in vitro ectomycorrhization with EN-98 and C-2, respectively, during a period of 6 months. All synthesized ectomycorrhizal seedlings were transplanted to 250-mL wide-mouth jars and acclimated for 4–7 months.

Cantharellus anzutake ectomycorrhization with pine and oak hosts by inoculation of previously established pine ectomycorrhizal systems

To establish a simple ectomycorrhization technique for *C. anzutake*, we used previously established pine ectomycorrhizal seedlings colonized with this fungus as

inocula for alternative hosts. Three different types of inocula were prepared for ectomycorrhization: mother plants, excised ectomycorrhizal root tips, and extraradical mycelia from acclimated soil. Non-mycorrhizal seedlings of *P. densiflora* germinated on a Petri dish (a few weeks old) and *Quercus serrata* grown in 250-mL wide-mouth jars filled with 200-mL autoclaved mineral soil (30–60 weeks old) were used as alternative hosts for ectomycorrhization. The oak seedlings were prepared from seeds sampled in the fall on the campus of the Faculty of Agriculture, Shinshu University.

An ectomycorrhizal pine seedling colonized with strain EN-61 and acclimated in a 250-mL wide-mouth jar (EN-61-12) for 1.5 years was transplanted to a straight-sided, wide-mouth polycarbonate jar with a volume of 1-L (Nalgene 2116-1000, Thermo Fisher Scientific, Waltham, MA) containing 1 L autoclaved mineral soil. Seven juvenile pine seedlings prepared as described above were also transplanted to the same jar. The plant jar was capped with an inverted jar, and the joint was sealed with polyvinylchloride film. The top jar had four vents (diameter, 5 mm), each of which was sealed with Milliseal. After ~ 4-month incubation, the jars were opened and the young pine seedlings were checked for ectomycorrhization. Alternatively established pine ectomycorrhizal seedlings were moved to a 250-mL jar system (EN61-12-2, EN61-12-4, EN61-12-6, and EN61-12-7; Supplementary Table 2), and seedling growth continued. The EN-61-12 mother plant also continued growth in the 1-L jar system.

Four ectomycorrhizal pine seedlings colonized with strain EN-51 and acclimated in 250-mL wide-mouth jars with 200 mL mineral soil (EN-51-5, EN-51-8, EN-51-9, and EN-51-10) were removed from the jars, and several long root systems (each several centimeters in length) that had developed ectomycorrhizal root tips were cut from the pine seedling using a pair of scissors. These ectomycorrhizal root systems were gathered in a Petri dish and the number of ectomycorrhizal root tips (i.e., ~ 1000) was roughly estimated. A 1-year-old non-mycorrhizal oak seedling was removed from the jar, inoculated with pine ectomycorrhizal root tips colonized with strain EN-51, and planted in a jar capped with an inverted jar (EN-51Q).

An ectomycorrhizal pine seedling colonized with strain EN-51 and acclimated in a 250-mL wide-mouth jar containing 200-mL mineral soil (EN-51-7) was removed from the jar, and a few long root pieces (each several centimeters in length) that had developed ectomycorrhizal root tips were cut from the pine seedling using scissors. These ectomycorrhizal root systems were gathered in a Petri dish, and the number of ectomycorrhizal root tips (i.e., ~ 200) was estimated. Three 7-month-old non-mycorrhizal oak seedlings were removed from their respective jars, inoculated with pine ectomycorrhizal root tips (50–70 tips per seedling) colonized with strain EN-51 by attaching both plant roots, and again planted in separate jars filled with mineral soil, each capped with an inverted jar (EN-51-7-1Q, EN-51-7-2Q, and EN-51-7-3Q, respectively).

Three ectomycorrhizal pine seedlings colonized with EN-51 and acclimated in 250-mL wide-mouth jars containing 200 mL mineral soil (EN-51-1, EN-51-2, and EN-51-13) were removed from their respective jars. Three 14-month-old non-mycorrhizal oak seedlings prepared as described above were then removed from their respective jars and transplanted into the jars that had contained the pine ectomycorrhizal seedlings colonized with EN-51; cultured soil containing extraradical EN-51 mycelium were also added to the jars. Each plant jar was again capped with an inverted jar (EN-51-1Q, EN-51-2Q, and EN-51-13Q, respectively).

All fungus-inoculated pine and oak seedlings in 1-L or 250-mL jars were placed in a biotron at 23 °C under continuous illumination by fluorescent light at 100 μmol m^{-2} s^{-1} and incubated for 3–10 months. The bottom jar was covered with aluminum foil to mask it from light. The plants were irrigated monthly and alternately with distilled water (30–100 mL) and the same volume of a solution of Hyponex 6–10–5 diluted to a ratio of 1:2000 with distilled water. After the incubation period, the root system of each seedling was observed, and the development of ectomycorrhizal root tips was confirmed. Seedlings with newly developed ectomycorrhizal systems were transplanted to fresh 1-L or 250-mL jars filled with mineral soil.

Scaled-up *C. anzutake* ectomycorrhizal systems

C. anzutake ectomycorrhizal systems of pine and oak hosts in 250-mL wide-mouth jars under non-sterile conditions (EN-51Q, EN-51-1Q–13Q, EN-51-7-1Q–3Q, C-2-7–10) were scaled up to straight-sided, wide-mouth 1-L polycarbonate jars (Nalgene 2116-1000) filled with autoclaved mineral soil. The soil surface within each plant jar was covered with polyvinylchloride film to protect soil moisture from evaporative loss in the growth cabinet. Thus, the ectomycorrhizal plants were grown in semi-open conditions. Plant jars were placed in a biotron at 23 °C under continuous illumination by fluorescent light at 100 μmol m^{-2} s^{-1} and incubated for 4–33 months. Jars were covered with aluminum foil to mask their contents from light. The plants were irrigated monthly and alternately with distilled water (~ 100 mL) and the same volume of a solution of Hyponex 6–10–5 diluted to a ratio of 1:2000 with distilled water. If *C. anzutake* fruiting structures occurred in the plant jars, their sizes were recorded and they were stored as dry specimens.

Each of the *C. anzutake* ectomycorrhizal systems with pine and oak hosts in 1-L wide-mouth jars under non-sterile and semi-open-pot conditions as described above or in 250-mL wide-mouth jars (EN-61-12-4–7 and EN-98-1–3) were scaled up to 4-L wide-mouth polycarbonate jars (Kitchen Pot, Endo

Shoji Inc., Tsubame, Niigata) filled with autoclaved mineral soil. Each plant jar was capped with an inverted jar, and the joint was sealed with polyvinylchloride film. The top jar had four vents (diameter, 5 mm), each of which was sealed with Milliseal. The bottom jar was covered with aluminum foil to mask its contents from light. Plant jars were placed in a growth cabinet in an experimental room at around 23 °C under continuous illumination at 150 μmol m^{-2} s^{-1} for 2 years. To monitor soil temperatures in relation to fruiting events, temperature probes were set in several jars at a soil depth of 10 cm and data were logged at 1-min intervals (DX100/DX200, Yokogawa Electric Corp., Tokyo). Irrigation was performed monthly and alternately with distilled water (100–200 mL) and the same volume of a solution of Hyponex 6–10–5 diluted to a ratio of 1:2,000 with distilled water.

As *C. anzutake* fruiting was observed repeatedly in the growth cabinet 4-L jar system during the subsequent 1.5 years, the soil temperature of these jars was controlled to maintain a stable temperature. We kept 10 4-L jars in a large water bath (water volume, ≈ 0.625 m^3) at a water temperature of 24 ± 0.5 °C for 2 months, after which the temperature was decreased to 20 ± 0.5 °C for 3 months. The temperature in the water bath was controlled using a water tank chiller (ZR-75E, Zensui, Osaka, Japan) and a circulation pump (Compact pump 2000, EHEIM GmbH & Co. KG, Deizisau, Germany). The remaining nine jars were placed in a different large water bath at a water temperature of 24 ± 0.5 °C for 3 months.

Microscopic characterization of fungal cell structures

During each seedling growth stage, ectomycorrhizal structures were observed in detail. A few ectomycorrhizal root tips were cross-sectioned longitudinally and mounted on glass slides using lactic acid. The mantle surface structure was observed in these slides using a differential interference contrast (DIC) microscope (AXIO Imager A1, Carl Zeiss) with a × 100 objective lens. Some ectomycorrhizal root tips were cross-sectioned transversally using a razor, and mounted on glass slides using lactic acid for Hartig net structure observation. Our descriptions of ectomycorrhizal structures followed the method of Agerer (2006).

We performed microscopic observation of all basidiomata and ectomycorrhizal root tips detected during our experiments. Basidia and basidiospore development was observed in the hymenial layers. The sizes of all basidiospores were measured.

Statistical analyses

Numbers of ectomycorrhizal root tips detected in in vitro experiments were compared between *C. anzutake* strains using one-way analysis of variance (ANOVA) with Kaleidagraph ver. 4.5 software (HULINKS Inc., Tokyo). Tukey's honestly significant difference (HSD) post hoc test ($P < 0.05$) was adopted to detect significant differences between means. For each inoculated *C. anzutake* strain, the numbers of mycorrhizal root tips detected in the ectomycorrhizal seedling acclimation experiment were compared between soil conditions using Student's *t* test ($P < 0.05$).

Molecular identification of growing ectomycorrhizal root tips and basidiomata

To determine whether growing ectomycorrhizal root tips were colonized with *C. anzutake* at each experimental step, the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA) of each sample was examined using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. DNA extraction and PCR amplification procedures were conducted according to Ogawa et al. (2018). PCR amplicons were then digested with *Hin*fI, *Hae*III, and *Rsa*I according to the manufacturer's recommendations.

Results

Ectomycorrhizal development in vitro

The five tested *C. anzutake* strains formed ectomycorrhizae on *P. densiflora* root systems (Table 2). In total, 87 (89%) of the 98 tested pine seedlings formed ectomycorrhizae. Ectomycorrhizae on the lateral root tips showed dichotomous branching and a yellowish fungal mantle (Supplementary Fig. 2a, d, g, j, m), which was similar to naturally sampled ectomycorrhizae of this fungus (Ogawa et al. 2019); however, the ectomycorrhizal root tips were shorter in length and brighter in color. The yellowish color of the fungal mantle was generally strong in strain EN-53 and weak in EN-51. The fungal mantle surface developed in two layers and was often cottony due to the distinct development of extraradical mycelium. Both the outer and inner layers indicated that the fungus was plectenchymatous, type B; however, the inner layer consisted of densely organized hyphae (Supplementary Fig. 2b, e, h, k, n). A clamp connection was often observed on each hyphal septum of the extraradical mycelium, and most hyphae contained oily, yellowish droplets. The oily droplets were often observed in organized mantle mycelia. Hartig nets developed in the root cortices down to the endodermis in all fungal strains tested (Supplementary Fig. 2c, f, i, l, o).

The number of ectomycorrhizal root tips differed between strains and was significantly higher in EN-52 than in EN-51, EN-60, or EN-61 (Table 2). Furthermore, seedlings inoculated with EN-52 had fewer total root tips than the other strains; therefore, the ectomycorrhizal colonization ratio was significantly higher in EN-52 than in all other strains.

Table 2 In vitro ectomycorrhization of *Cantharellus anzutake* with pine seedlings

Strain ID	Number of seedlings:		Incubation period (day)	Means with standard errors in parentheses ($N = 20$ or 19)[1]					
	Tested	Ectomycorrhized		Ectomycorrhizal root tips per seedling[1]		Total root tips per seedling[2]		Ectomycorrhizal colonization (%) of root tips	
EN-51	20	13	300	24.9 (5.8)	c	1009 (101)	a	3.9 (1.1)	b
EN-52	20	20	240	95.7 (10.3)	a	582 (49)	b	16.9 (1.5)	a
EN-53	19	18	305	81.6 (16.7)	ab	1161 (89)	a	7.7 (1.3)	b
EN-60	19	18	315	41.6 (7.1)	bc	938 (94)	a	6.1 (1.4)	b
EN-61	20	18	320	43.8 (10.6)	bc	1130 (94)	a	9.6 (2.5)	b

[1] Different letters in a column indicate significant differences ($P < 0.05$, Tukey's HSD post hoc test)

[2] Estimated counts from 1/8 of the entire root system

Acclimation of ectomycorrhizal pine seedlings under non-sterile conditions and different soil conditions

A total of 79 pine ectomycorrhizal seedlings synthesized with *C. anzutake* were tested for ectomycorrhizal development in non-sterile conditions with different soil types (Table 3). In total, 66 (84%) ectomycorrhizal seedlings retained and developed ectomycorrhizae, whereas 13 seedlings exhibited no ectomycorrhizal root growth during incubation. In the latter seedlings, no fresh ectomycorrhizal root tips were observed at the end of the incubation period. Ectomycorrhizal root tips developed under non-sterile conditions were generally more slender (Fig. 1a, b, d, e) than those synthesized in vitro (Supplementary Fig. 2), but quite similar to those observed in naturally occurring ectomycorrhizae (Ogawa et al. 2019).

The proportion of seedlings that retained and developed target ectomycorrhizae under non-sterile conditions was similar in mineral soil (92%) and organic soil (80%) ($P = 0.340$, *t* test; Table 3). In terms of plant growth, ectomycorrhizal seedlings had significantly more needles when grown in organic soil than in mineral soil among all tested *C. anzutake* strains. The needles of pine seedlings grown in organic soil exhibited a strong green color (Fig. 1c, f). We counted the mycorrhizal root tips of seedlings inoculated with strains EN-53 and EN-60 and found significantly more ectomycorrhizal root tips inoculated with EN-53 in organic soil (Table 3). There were also more ectomycorrhizal root tips in EN-60-inoculated seedlings in organic soil; however, this difference was not significant, perhaps due to the limited number of seedlings measured. Pine seedlings colonized with the other *C. anzutake* strains showed similar trends in growth and ectomycorrhizal development (Fig. 1d–f).

Table 3 Fruiting of *Cantharellus anzutake* and host pine growth under non-sterile conditions in 250-mL jars

Strain	Mineral soil condition					Organic soil condition				
	Number of seedlings			Means with standard errors in parentheses		Number of seedlings			Means with standard errors in parentheses	
	Tested	With target EM	With fruiting	Needle number per seedling[1]	EM root tip number per seedling[2]	Tested	With target EM	With fruiting	Needle number per seedling[1]	EM root tip number per seedling[2]
EN-51	8	7	0	13.6	ND	5	1	0	32	ND
EN-52	9	7	0	21.6 (3.0)*	ND	9	5	0	68.6 (3.0)	ND
EN-53	8	8	0	10.0 (1.4)*	358 (61)*	10	10	0	65.2 (5.3)	1346 (62)
EN-60	4	4	1	14.3 (2.3)*	634 (170)**	8	8	0	48.6 (3.7)	746 (122)
EN-61	10	10	2	13.8 (2.6)*	248	8	8	0	55.3 (6.0)	ND

ND not determined

[1] This value was calculated for seedlings that retained or developed target ectomycorrhizae (EM). Data were compared between soil conditions in each fungal strain by the *t* test. *Significant at $P < 0.01$

[2] This value was calculated for limited numbers of seedlings: 6 for EN-53 with mineral soil, 4 for EN-53 with organic soil, 2 for EN-60 with mineral soil, 4 for EN-60 with organic soil, and 1 for EN-51 with mineral soil. The number of EM root tips was not counted in the other seedlings. The data were compared between soil conditions in each fungal strain by the *t* test. *Significant at $P < 0.05$. **Not significant at $P = 0.05$

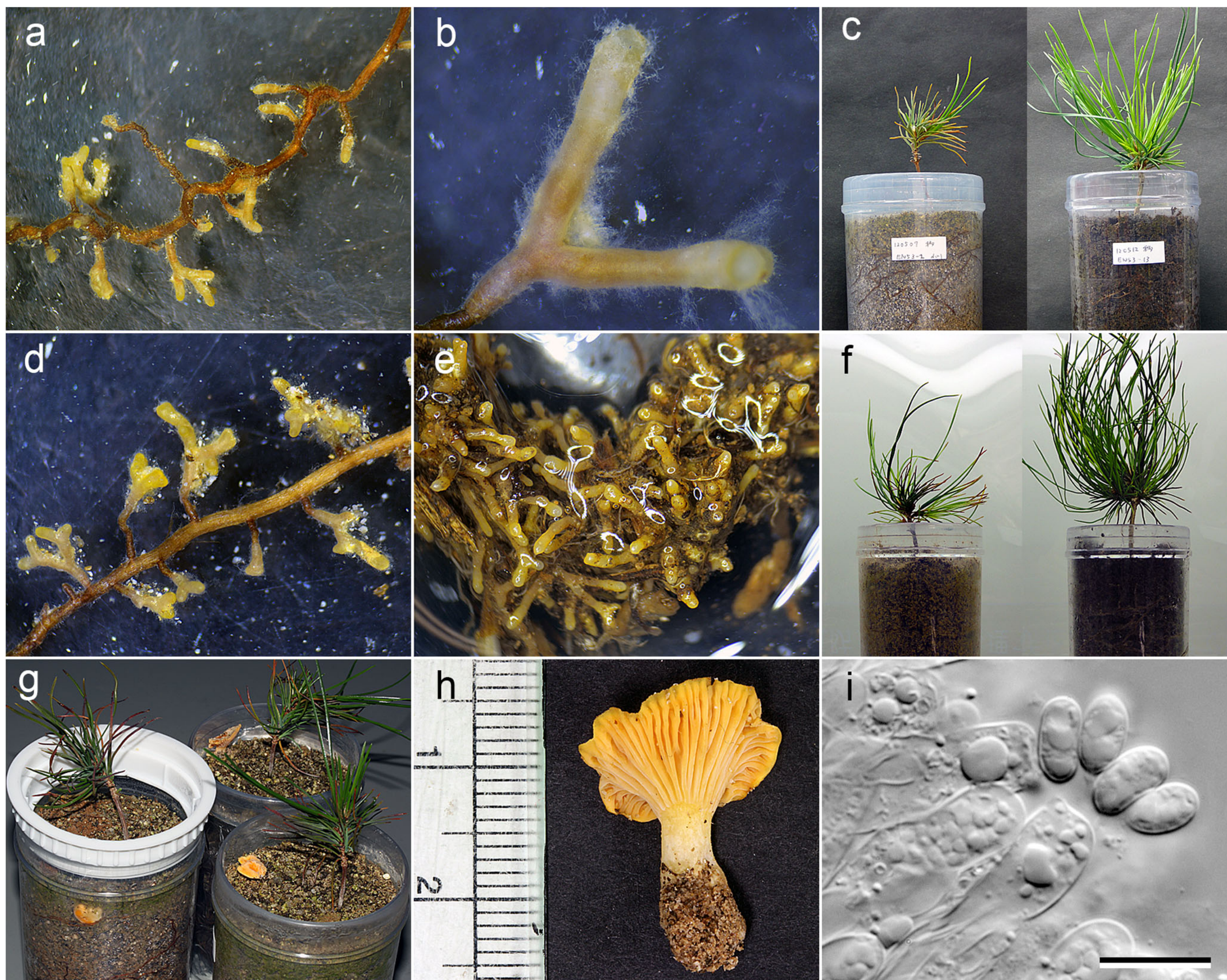

Fig. 1 Acclimated ectomycorrhizal system of *Cantharellus anzutake* in wide-mouth 250-mL jar with different soil conditions and basidiomata fruiting. Strains EN-51 (**a**–**c**) and EN-61 (**d**–**f**). Ectomycorrhizal root system developed in mineral soil (**a**). Ectomycorrhizal root tips with typical dichotomous branching developed in mineral soil (**b**). Comparison of shoot growth of host pines grown in mineral soil (left) and organic soil (right) (**c**). Ectomycorrhizal root system developed in mineral soil (**d**). Ectomycorrhizal root tips developed in organic soil, showing a densely grown ectomycorrhizal root cluster (**e**). Comparison of shoot growth of host pines grown in mineral soil (left) or organic soil (right) (**f**). Fruiting of basidiomata in jars of EN-60-10 (left), EN-61-10 (right, rear), and EN-61-9 (right, front) (**g**). Developed hymenium of a basidioma sampled from EN-61-10 (**h**). Basidium with four basidiospores observed on basidioma sampled from EN-60-10 (**i**). Micrograph of basidium (**i**) constructed from two photographs with the same field of view but different focus depths (ca. 2 μm) using the Adobe Photoshop CC Synthetic function. Bar, 10 μm

All ectomycorrhizal root tips analyzed by PCR-RFLP showed identical electrophoresis patterns to those of inoculated mycelia (data not shown).

Cantharellus anzutake ectomycorrhization with pine and oak hosts by inoculation of previously established pine ectomycorrhizal systems

Strain EN-61 colonized on alternative pine seedlings (EN-61-12-2, EN-61-12-4, EN-61-12-5, EN-61-12-6, and EN-61-12-7) via the mother plant technique, and about 10 ectomycorrhizal root tips were observed on each juvenile seedling, with fewer than 100 total root tips/seedling. Strain EN-51 colonized on alternative oak seedlings (EN-51Q, EN-51-1Q, EN-51-2Q, EN-51-13Q, EN-51-7-1Q, EN-51-7-2Q, and EN-51-7-3Q) after inoculation of ectomycorrhizal root tips or soil containing extraradical mycelium of this fungal strain, and several hundred ectomycorrhizal root tips/seedling were observed in the root systems of the oak seedlings (Supplementary Fig. 3). PCR-RFLP patterns in the fungal ITS regions of the ectomycorrhizal root tips sampled form several seedlings were identical to those of the original cultured mycelial strains (data not shown).

Fruiting of *C. anzutake* in the 250-mL jar system with mineral soil

Four months after transplantation of in vitro synthesized ectomycorrhizal pine seedlings to the 250-mL jar system, one seedling colonized with EN-60 (EN-60-10) and two seedlings colonized with EN-61 (EN-61-9, EN-61-10) exhibited fruiting with yellow to yellow-orange basidiomata (Table 3, Fig. 1g, h). Although EN-60 basidioma occurred via hypogeous fruiting (Fig. 1g), microscopic observation revealed distinct basidiospore formations, 6.8–8.7 (7.8) × 3.9–5.1 (4.5) μm in size (Fig. 1i). Basidiomata of E-61 exhibited epigeous fruiting, with funnel-shaped pileus (diameter, 1.0–1.5 cm), cylindrical stipe tapering slightly at the apex (width, 3.7–6.0 mm; length, 1.0–1.5 cm), and basidiospores (6.0–8.2 [7.3] × 3.6–5.1 [4.5] μm). Spore size and shape were identical to those provided in our previous species description (Ogawa et al. 2018).

The occurrence of *C. anzutake* basidiomata was observed only in jars filled with mineral soil (Table 3, Fig. 1g–i). The growth measurements of host plants in these jars were not significantly different from those of hosts in non-fruiting jars with mineral soil (data not shown).

Fruiting of *C. anzutake* in the 1-L and 4-L jar systems with mineral soil

Although *C. anzutake* fruiting was also observed in the 1-L EN-51/oak seedling jar (EN-51Q; Supplementary Fig. 3c), fruiting did not occur in other 1-L jars. Plant shoot growth, especially needle and leaf development, was under stress in the semi-open jar system, likely due to the greater evaporation and drier conditions in the growth cabinet (Supplementary Fig. 3a, b). In contrast, fruiting was observed in 12 of the 4-L jars (closed-jar system), representing 60% of all tested jars (Table 4). The first flush of a basidioma (EN-51-13Q) was observed 5.5 months after transplantation of the mycorrhizal seedling to the 4-L jar. This basidioma was significantly larger than those occurring in the 250-mL jars, and cream-colored spore deposits were observed on the soil surface (Fig. 2a). The largest basidioma found in a 4-L jar (EN-61-12; Fig. 2b, c) was 4.5 cm in pileus width, and 2.2 cm in stipe length, which is considered a moderate size for this fungus in nature (Ogawa et al. 2018). Most basidiomata observed in the 4-L jar system were epigeous-fruiting, pale yellow to orange-yellow on the pileal surface, sometimes with a weak apricot smell, showing a cream-colored spore print on the soil surface in fully matured cases. Strain C-2 showed a paler color in the young basidioma stage (Fig. 2d).

In total, fruiting events were observed 29 times among all fungal strain–host species combinations (Table 4). Of these, the EN-61-12 jar flushed with basidiomata nine times, and another six jars flushed at least twice. Because these fruiting events were discontinuous throughout the incubation period, we speculate that there is a relationship between temperature and flushing. Soil temperature fluctuated in the 4-L jars with room temperature, because the air conditioning system in the room switched between cooling and heating in spring and fall, respectively (Fig. 3). Under such fluctuating soil temperatures, EN61/pine combination jars produced fruit 12 times (Table 4, Fig. 3). In the 30 days immediately prior to basidiomata maturation (i.e., fully opened pileus observed) soil temperatures ranged from 19 to 27 °C, mainly within the narrower range of 23–25 °C. In the water bath system used to maintain stable soil temperatures in the 4-L jars, fruiting occurred at both 20 and 24 °C (Table 4).

Table 4 Fruiting of *Cantharellus anzutake* in the tested 4-L jars with ectomycorrhizal hosts

Strain / host		EN-51 / Oak							EN-61 / Pine						C-2 / Pine				EN-98 / Pine		
ID of planted jar		Q	1Q	2Q	13Q	7-1Q	7-2Q	7-3Q	12	12-2	12-4	12-5	12-6	12-7	7	8	9	10 [1]	1	2	3
Year / Month		Date of fruiting during year/month in each 4-L jar																			
2015	Apr				2				23												
	Jul											9									
	Aug																			26	
	Sep								7		7					25	7				
	Dec									1		1									
2016 [2]	Jan								7			14				14				7	
	Mar				23				29											23	
	Apr		25																		
	May								26												26
	Jun								28	2										29	
	Jul	28																			
	Aug								18												18
	Oct								24												
	Dec								20										16		

[1] This seedling was removed from this experiment in February 2016 and used in another experiment

[2] Orange and blue highlighted areas indicate incubation of the bottom jars in water baths at 24 °C and 20 °C, respectively

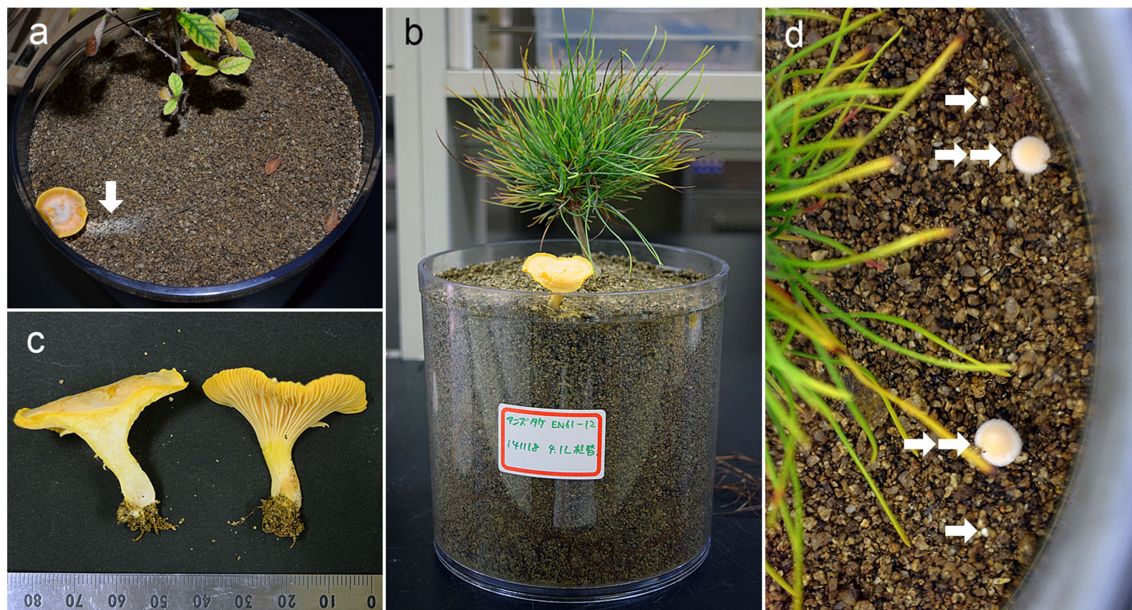

Fig. 2 Fruiting of *Cantharellus anzutake* in a 4-L jar. Basidioma of strain EN-51 associated with *Quercus serrata* host (EN-51-13Q) showing spore deposit (arrow) on the jar soil surface (**a**). Basidioma of strain EN-61 associated with *Pinus densiflora* host (EN-61-12; **b**, **c**). Young basidiomata (double arrow) and primordia (single arrow) of strain C-2 associated with *P. densiflora* host (**d**)

Discussion

This is the second report of chanterelle fruiting as a result of mycorrhizal synthesis under controlled environmental conditions. The fruiting of European yellow chanterelle in a controlled environment was first reported over two decades ago (Danell and Camacho 1997), in which small but distinct *C. cibarius* basidomata were observed to flush several times from potted ectomycorrhizal pine seedlings in a greenhouse (Pilz et al. 2003). Our study focused on the cryptic Japanese yellow chanterelle *C. anzutake*, formerly identified as *C. cibarius* s.l. (Ogawa et al. 2018). Although our study was not the first to report chanterelle fruiting under such conditions, we achieved repeated fruiting events using several different culture strains, different host species and ages, and different magnitudes of the fungus–host association (i.e., according to seedling size and soil volume). These successful *C. anzutake* fruiting events will provide insight into the cultivation of chanterelles and other edible ectomycorrhizal mushrooms.

The first fruiting of *C. anzutake* occurred during the fifth month of acclimation of pine mycorrhizal seedlings under non-sterile conditions, when the host plants were 15 months old and around 10 cm in shoot height. The fruiting of edible ectomycorrhizal mushrooms with young, small hosts was also reported for *Rhizopogon roseolus* (Corda) Th. Fr. and *Lactarius akahatsu* Nobuj. Tanaka (Yamada et al. 2001), both of which are categorized as early-stage ectomycorrhizal fungi in terms of forest tree succession. In nature, *C. anzutake* fruiting occurs in closed-canopy forests, suggesting that it is a late-stage fungus. However, its fruiting habitats are not deep forest, but young, growing host plants (Ogawa et al. 2018). Therefore, *C. anzutake* may have ecophysiological potential as an early-stage ectomycorrhizal fungus. The ability to fruit with a young seedling confers a practical advantage to *C. anzutake* and probably to *C. cibarius* in terms of spore generation for mushroom breeding. However, our preliminary trials to establish spore isolates from *C. anzutake* basidiomata produced in the 4-L jar system were not successful, although the size and shape of basidiospores observed on these basidiomata were almost identical to those of naturally occurring basidiomata. It is worth considering that *C. anzutake* basidiomata that fruited in the 4-L jars involved endofungal microbes (unpublished data), as in naturally sampled basidiomata (Ogawa et al. 2019). This phenomenon was probably caused by microbial colonization from the surrounding soil and may have ecophysiological significance for chanterelle reproduction.

Primordium induction for fruiting is one of the main steps in mushroom cultivation (Miles and Chang 2004). We first tested two soil types for ectomycorrhizal development under non-sterile conditions using a 200-mL soil volume; we expected better mycorrhizal development in organic andosol soil because it contained higher levels of nitrogen and phosphorus than the tested granite-based mineral soil (Saito et al. 2018). Indeed, andosol soil yielded significantly better pine shoot growth (Table 2), with some pine seedlings showing densely clustered ectomycorrhizal root tips in the potted soil (Fig. 1). However, fruiting was

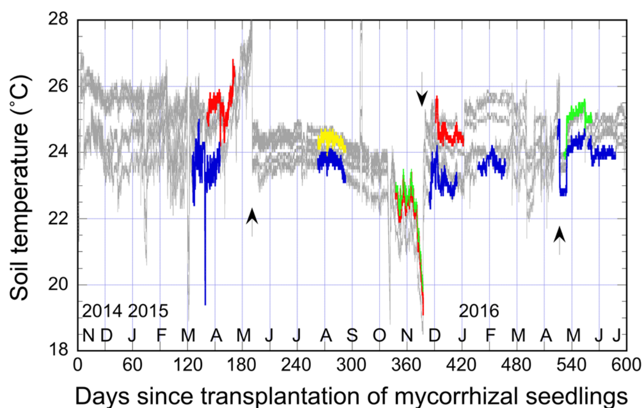

Fig. 3 Soil temperatures in six 4-L jars of pine hosts colonized with the EN-61 strain of *Cantharellus anzutake*. Gray lines indicate measured temperatures in the six jars (see Table 4). Blue, green, yellow, and red lines indicate temperatures in the EN-61-12, EN-61-12-2, EN-61-12-4, and EN-61-12-5 jars, respectively, 30 days before fruiting in the jars. Arrows indicate the time at which the laboratory air conditioning system switched between heating (down) and cooling (up)

observed only in mineral soil (Table 3, Fig. 1). This fruiting phenomenon suggests that soil nutrient deficiency in the plant jars could activate a specific metabolic pathway to support primordium morphogenesis of this fungus. Thus, nutrient-poor conditions, which can signal starvation, may promote specific metabolic activity for reproduction to survive through fruiting and spore dispersal (Skromne et al. 1995; Donofrio et al. 2006; Palmer and Horton 2006; Zhang et al. 2015; Kües et al. 2016; Liu et al. 2016). When mycelia develop in association with a symbiotic host under such a physiological state, environmental signals can function as triggers to begin primordium morphogenesis. The 4-L jar system showed first fruiting 6 months after the transplantation of mycorrhizal seedlings (Table 4). This result can be explained by the initial development of a mycorrhizal root system in transplanted mycorrhizal seedlings in the 4-L jar system, followed by increased mycelial biomass in the mineral soil, and the response of physiologically activated mycelium to induction signals for primordium morphogenesis.

Induction signals, including temperature, for primordium morphogenesis have been well studied in cultivated mushrooms (Eastwood et al. 2013; Sakamoto 2018). Among ectomycorrhizal fungi, temperature fluctuation has been suggested as an important trigger for fruiting in nature (Hamada 1953; Kinugawa 1963). The first fruiting of *C. anzutake* in the 250-mL jar system was observed under growing conditions at 23–25 °C under 24 h of continuous lighting. Therefore, we adopted this condition for the up-scaled 4-L jar system. Throughout 1.5 years of monitoring the 4-L jars, soil temperatures fluctuated between 18 and 28 °C even under constant air conditioning at 23 °C in the experimental room. This fluctuation exerted a temperature effect on chanterelle fruiting. *C. anzutake* fruiting in nature occurs from spring to fall depending on the geographic region, and common meteorological properties include daily mean temperatures of 20–25 °C and humid, rainy weather, which roughly matches the conditions within the 4-L jar system. Because fleshy Agaricomycetidae basidiomata take 2–3 weeks for maturation from a small primordium knob, we examined soil temperatures for the 30 days prior to basidioma observation in the 4-L jars, and detected a fruiting induction trend at temperatures of around 23–25 °C (Fig. 3). Therefore, we suggest that this temperature range is a signal for *C. anzutake* fruiting. An additional reciprocal experiment, with 4-L jars placed in water baths at 20 and 24 °C at 2-month intervals, revealed a wider temperature range for fruiting induction over 20 °C. Known induction temperatures for fruiting of ectomycorrhizal basidiomycete mushrooms are in fact quite limited among measured data (Kinugawa 1963; Debaud and Gay 1987; Ohta 1994), although phenological patterns in nature suggest its significance in diverse wild mushrooms (Imazeki and Hongo 1989; Pilz et al. 2003; Kauserud et al. 2008, 2012). In *Tricholoma matsutake*, induction temperatures for fruiting were measured in situ, and found to be approximately 19 °C and lower at soil depths of 5–10 cm (Kinugawa 1963, Ogawa 1978; Furukawa et al. 2016; Vaario et al. 2017). As *T. matsutake* develops meter-sized mycelial areas (*shiro*) within the soil mineral layer, detailed soil temperature measurements can be obtained for this species even in natural forest conditions. However, soil temperature data are difficult to obtain for most ectomycorrhizal fungi during the production of individual basidiomata in situ. Therefore, fruiting experiments with hosts maintained under controlled environmental conditions can allow researchers to examine the effects of temperature on fruiting induction in edible ectomycorrhizal mushrooms.

In the present study, the observation of different fruiting patterns in *C. anzutake* under different host plant species and age conditions, and in jars of different sizes, suggests the importance of these factors for successful fruiting. The EN-61/pine line in the 4-L jars strongly suggests the importance of host plant age, because a single 4–5.5-year-old pine host (incubation period of the 4-L jar experiment) fruited more densely than 1.5–3-year-old pine hosts (Table 4). This phenomenon can be explained by the structure of the root system, as well as the probable increased carbohydrate supply by an older host. We cut the young roots of the older host plant EN-61-12 during each transplantation process (into 250-mL, 1-L, and 4-L jars) due to coiling, excessive root growth at the bottom of the jars. Therefore, the growing root system in the 4-L jar soil consisted of large and multi-aged root parts. These heterogeneities within the root system and probable larger magnitude of carbohydrate supply may have signaled the associated *C. anzutake* mycelium to induce fruiting.

We tested the association of oak hosts with EN-51 (originally obtained from an oak forest) in the 4-L jar experiment, because this strain did not fruit with pine hosts in the 250-mL jar

system. The other three strains used in the 4-L jar experiment with pine hosts were originally obtained from conifer forests, although two of these had natural and in vitro hosts that differed at the genus or species level (Tables 1 and 4). However, no significant differences were observed among the basidiomata of the four strains tested. Therefore, *C. anzutake* strains tested in the present study were fully compatible with oak and pine for both ectomycorrhizal formation and fruiting, i.e., a complete generation. Among EN-51-associated oak seedlings, three of four that germinated in 2010 provided *C. anzutake* basidiomata; however, three others that germinated in 2011 did not (Table 4). This result was probably related to either the genetic background of the tree seeds used or the host plant age, as discussed above. Of the four strains tested in the 4-L jar experiment, EN-51, EN-61, and EN-98 were isolated from forests with the same climate, i.e., cool temperate regions. The pine (*P. densiflora*) and oak (*Q. serrata*) hosts are common species for this climate, suggesting an ideal combination for ectomycorrhization with these strains. In contrast, strain C-2, which was isolated from an ectomycorrhizal root tip of *P. luchuensis* in a subtropical region (Table 2), also fruited with a *P. densiflora* host. Therefore, practical chanterelle cultivation can be achieved under controlled environmental conditions beyond fungal ecological traits such as the climate within its natural habitat, as long as the tested fungal strains are predisposed to exotic hosts and established ectomycorrhizal status.

Jar size was closely linked to host plant size and basidioma size among the 250- to 4-L jars: the maximum diameter of a basidioma pileus was over twice as large in the largest jars (Figs. 2 and 3). This result is explained by carbon flux to the ectomycorrhizal fungus from the host (Smith and Read 2008). Thus, scaling up the jar system will provide larger *C. anzutake* fruit and more frequent fruiting. The 4-L jar system may be sufficient for harvesting medium-sized mushrooms in cultivation. As pine seedlings in association with *C. cibarius* have been observed to grow quickly under CO_2 exposure at 2,000 ppm due to increased photosynthetic rate (Danell 1994), such gas control in our experimental system could accelerate or increase mushroom fruiting in future studies. Thus, the nutrient balance between these symbiotic organisms must be taken into account for the benefit of ectomycorrhizal biomass and the fruiting induction mechanism. In the current study, our 4-L jar system yielded only 29 *C. anzutake* fruiting events from 20 host seedlings (mean, 1.45 times/jar) within 2 years of observation. If the mean value increased to the maximum value recorded in the EN-61-12 jar, fruiting would be observed around 180 times in 20 jars. Therefore, optimizing the mycorrhizal system for fungal artificial cultivation through the selection of highly productive fungal strains would yield greater value.

In conclusion, we succeeded in ectomycorrhizal synthesis of the Japanese yellow chanterelle *C. anzutake* in vitro with pine hosts, and acclimated these ectomycorrhizal seedlings under non-sterile conditions. We also achieved repeated fruiting of this fungus in a jar system under controlled environmental conditions and partly specified fruiting conditions. The data collected in this study will promote further chanterelle cultivation studies.

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Compliance with ethical standards

All experiments were performed in compliance with the current laws of Japan.

Conflict of interest The authors declare that they have no conflicts of interest.

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