Effect of Aging on Culture and Cultivation of the Culinary-Medicinal Mushrooms *Morchella importuna* and *M. sextelata* (Ascomycetes)

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ABSTRACT: The prominent problem of "uncertainty" has been frustrating morel farming since the commercial production of *Morchella* mushrooms was realized in 2012 in China. Spawn aging may be the main underlying reason. In this paper, aging in cultivated strains of *M. importuna* T4 and *M. sextelata* T6 was achieved by successive subculturing. Oxidative stress, mycelial growth rate, biomass, sclerotial formation, pigmentation, and yield of different subcultures were determined. The results suggested that *M. importuna* T4 and *M. sextelata* T6 exhibited systemic senescence manifested as 24 and 17 subcultures and lifespan of 3048 and 2040 h, respectively. Aging showed a close relevance to oxidative stress. Regression analysis revealed a strong positive correlation between time of subculturing (aging) and lipid peroxidation (oxidative stress) in both morels. In addition, pigmentation tended to increase, while the number of sclerotia tended to decrease, with the rise of subculturing times in both morels. Moreover, the mycelial growth rate and biomass of the last two subcultures were significantly lower than those of others, indicating that cultural characteristics may be used as signs of seriously aging culture. Finally, the yield of subcultures was significantly lower than that of the original strains in artificial cultivation. Regression analysis showed a strong negative correlation between time of subculturing and yield in two morels. This work will improve the understanding of "uncertainty" and thus be beneficial for stable development of morel farming.

KEY WORDS: field cultivation, medicinal mushrooms, *Morchella importuna*, *M. sextelata*, morel, mycelial growth, oxidative stress, successive culturing

ABBREVIATIONS: BHA, butylated hydroxyanisole; **CYM**, complete yeast extracts medium; **EDTA**, disodium ethylenediaminetetraacetate dihydrate; **HCl**, hydrochloric acid; **HSD**, honestly significant difference; **MDA**, malondialdehyde; **SD**, standard deviation; **TBA**, thiobarbutic acid; **TCA**, trichloracetic acid

I. INTRODUCTION

Mushrooms have been valued by humankind as edible and medicinal resources for more than a thousand years. Traditional use of medicinal mushrooms has been long established among different ethnic groups.[1,2] *Morchella* mushrooms (Pezizales, Ascomycota) have been used as traditional foods and folk medicines to treat excessive sputum, gastrointestinal disorders, and shortness of breath in China, India, and Pakistan.[3–5] As culinary mushrooms, true morels (ascocarps of the mushroom) are highly prized for their appearance as well as their high-quality taste and aroma; they are intensively picked and widely sought-after around the world.[6,7] Meanwhile, studies of bioactive substances suggested that morels possess antiinflammatory, antitumor, antioxidant, antiviral, antimicrobial, antileishmanial, neuritogenic, hepatoprotective, and immunostimulatory properties.[7–13] The prominent nutritional and medicinal values inspired pervasive fundamental and technological studies for commercial cultivation of *Morchella* mushrooms to meet the still-increasing demands for morels by customers for more than a century.[6,14] Ower[15] innovatively achieved morel indoor cultivation, which enabled morel commercial cultivation to be realized.[15] However, the complexity of applied conditions and the involvement of the use of *M. rufobrunnea* restrict the application and popularization of

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Ower's technology.[16,17] On the basis of Ower's innovations, artificial cultivation of *Morchella* mushrooms in field has been realized since 2012 in China.[6,14] In the production season of 2017–2018, the total cultivation area exceeded 4600 ha in most areas of China. Some strains of *M. importuna* (e.g., 1#, 3#, 4# etc.) and *M. sextelata* (e.g., 6#, 13# etc.) have been widely applied in scale outdoor production.[14,18,19]

The main problem of *Morchella* outdoor cultivation is its "uncertainty." The average yield can reach 1500 ~ 2250 kg fresh morel per ha for experienced farmers. Occasionally, the yield may exceed 6000 kg per ha in small-scale cultivation. However, failure of production is also common. Statistics showed that the cultivation of about 70% of farmers is of poor profitability every year, with yield less than 1500 kg per ha. Some fundamental studies on *Morchella* (e.g., nuclear behavior during meiosis and ascosporogenesis,[18] reproductive strategies and mating type,[20–23] as well as effects of material formula,[24] surface fire,[25,26] and microelement[27,28] on cultivation) may promote the development of morel artificial cultivation. Nevertheless, there are many confusing problems to be settled regarding genetics of vegetative incompatibility, mechanism of fructification, spawn aging and degeneration, as well as role of conidia; these knowledge gaps restrict the healthy and long-term development of morel farming.[19]

Aging is a time-related process of progressive decline in the ability to withstand stress, damage, and disease.[29] A loss or reduction in sporulation and virulence was found in some senesced strains of entomopathogenic fungi[30,31] and thus may cause great loss for production of biological pesticides. Experience shows that application of aging spawns in cultivation may bring remarkable losses for morel production.[32] Spawn aging may be the main reason underlying "uncertainty" of morel farming. However, there has been no scientific conclusion. In this study, the cultural and cultivated characteristics of aging strains of *M. importuna* and *M. sextelata* were determined. This work uncovered the effect of aging on morel culture and cultivation, and thus will promote the breeding and spawn production of *Morchella* mushrooms and be beneficial for the stable development of the morel industry.

II. MATERIALS AND METHODS

A. Mushroom Strains and Successive Subculturing

Strains of *M. importuna* T4 and *M. sextelata* T6 are production strains domesticated from wild morels in Sichuan Province, China. They are available from Peixin He in Zhengzhou University of Light Industry and Wei Liu in Huazhong Agricultural University, China. The strain obtained via tissue separation from fresh ascocarp represents subculture 0 (T4-0 or T6-0). Successive subculturing was done by taking inoculum from the subculture 0 to produce the 1st subculture (T4-1 or T6-1), repeated for the 2nd one (T4-2 or T6-2) from the 1st, and so on.[32,33] Briefly, a 2-mm–diameter mycelial plug from stock culture was inoculated on one side of a Petri dish (9 cm diameter) of complete yeast extracts medium (CYM) (glucose 20 g/L, yeast extracts 2 g/L, peptone 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.46 g/L, and agar 20 g/L) and incubated at 24°C in the dark. Before the mycelia reached the other side of a Petri dish, mycelial plugs containing the whole hyphal tips excised from colony edges were transferred to fresh plates with growth tips toward the margin of medium and continued to cultivate until growth cessation. Meanwhile, the adjacent plugs close to old inoculum of plate cultures were transferred to CYM slant and then incubated at 24°C in the dark. The slant cultures were preserved at 4°C for short-term use or –80°C under the protection of 15% (v/v) glycerol solution for long-term use. Each subculture was repeated three times with inocula of the same age and from the same plate.

B. Assay of Cultural Characteristics

In successive subculturing, mycelia growth ceased in the 24th subculture of *M. importuna* T4 and 17th subculture of *M. sextelata* T6. Therefore, subcultures of T4-0, -3, -6, -9, -12, -15, -18, -21, and -23 of *M.*

importuna, and T6-0, -2, -4, -6, -8, -10, -12, -14, and -16 of *M. sextelata* were selected for further study. For assay of cultural characteristics, mycelial plugs (4 mm diameter) from respective slant culture were placed in the center of CYM plates covered with autoclaved cellophane membranes.[34] The inoculated plates were incubated at 24°C in the dark for 14 d. The mycelial growth rate was calculated when the mycelial tips reached the other side of a Petri dish by the following formula: mycelial growth rate (mm/h) = (radius of colony – 2)/incubation hours. The sclerotial development was observed. The biomass (g/d) was calculated by dividing total time of cultivation (14 d) by the fresh weight of mycelia and sclerotia on cellophane membrane. Each culture was repeated three times with inocula from the same slant.

C. Assay of Lipid Peroxidation

Oxidative stress in growing cultures was tested by measuring lipid peroxidation by a modified thiobarbutic acid (TBA)-based method.[34,35] Specifically, the fresh mycelia on the cellophane membrane of 14-day plate cultures were homogenized and 0.5 mL of homogenate was mixed with 0.5 mL TBA reagent [0.5% (w/v) TBA in 20% (w/v) trichloracetic acid (TCA) and 0.33 N hydrochloric acid (HCl)]. Five microliters of 2% (w/v) of the lipid antioxidant butylated hydroxyanisole (BHA) (in absolute ethanol) was added to the resulting mixture to prevent artificial lipid peroxidation during the assay. The mixture was incubated at 100°C for 20 min, added with 1 mL butanol, mixed by vigorous vortexing, centrifuged at 15,000 *g* for 3 min, and its absorbance was measured at 535 and 600 nm against a sample blanks (0.5 mL phosphate-disodium ethylenediaminetetraacetate dihydrate [EDTA] buffer, plus 0.5 mL TBA reagent containing 0.02% w/v BHA). The absorbance difference ($A_{535} - A_{600}$) was converted to malondialdehyde (MDA) equivalents using the extinction coefficient for MDA of 1.55×10^5 $M^{-1}cm^{-1}$. Lipid peroxidation was expressed in mmol MDA/g fresh mycelia.

D. Outdoor Planting Experiment

The outdoor planting experiments were conducted in the production season of 2016–2017 by technology of field soil cultivation in Suyahu Wetland Reserve, western suburb of Zhumadian City, Henan Province, China (114.02E, 32.98N). The cultivation of each isolate was repeated three times in different plastic canopies. The area of each small plot was about 15 m^2. The whole process of morel artificial cultivation can be roughly divided into six major stages: spawn production, land preparation, spawning, exogenous nutrition supplying, fruiting management, and harvesting.[14] The spawning time was on November 4, 2016. The dosage of seeding (0.5 kg/m^2) and exogenous nutrition (1.2 kg/m^2) and other management measures were the same. The mature ascocarps were harvested and weighed until the end of the production season (before April 15, 2017).

E. Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance followed by the Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$). Pearson regression analysis was used for correlation assessments. All analyses were carried out using IBM SPSS Statistics 22.

III. RESULTS

A. Systemic Senescence through Successive Subculturing

M. importuna T4 and *M. sextelata* T6 exhibited systemic senescence within a distinct time frame through successive subculturing. The lifespan (life expectancy) on CYM was from 2040 h (T6) to 3048 h (T4) (Fig.

1). *M. sextelata* T6 is apt to age on CYM plate in comparison with *M. importuna* T4. The subculture times were 17 for strain T6 and 24 for T4 (Fig. 1). Moreover, although the total growth length of *M. sextelata* T6 (121.1 cm) was shorter than that of *M. importuna* T4 (132.1 cm), their difference (9.08%) was mild compared with subculture times (41.2%) and lifespan (49.4%), because the mycelia of T6 grew faster than T4 (Fig. 1). The lifespan can be roughly divided into juvenile phase and senescent phase with respect to mycelia linear growth rate.[32] Compared with *M. importuna* T4, the period of senescent phase of *M. sextelata* T6 was longer (408 h vs. 192 h), but the juvenile phase was shorter (1632 h vs. 2856 h). Subculture of T4-23 and T4-24 of *M. importuna* and that of T6-15, T6-16, and T6-17 of *M. sextelata* were at the senescent phase (Fig. 1).

B. Correlation between Time of Subculturing and Lipid Peroxidation

Lipid peroxidation is a common differentiation-associated indicator of oxidative stress. In particular, the levels of MDA are used as a measure of the degree of this stress.[36] MDA assay of different subcultures suggested that the levels of MDA were overall increased with the rise of subculturing time (Tables 1 and 2). Regression analysis showed a significantly positive correlation between time of subculturing and MDA in *M. importuna* T4 ($r = 0.937$, $P = .000$) and *M. sextelata* T6 ($r = 0.787$, $P = .012$). The results definitely suggested that morel aging was closely relevant to oxidative stress. Nevertheless, the detailed MDA variation of subcultures in *M. importuna* T4 and *M. sextelata* T6 was different. In *M. importuna* T4, the difference of lipid peroxidation of the original strain and the 1st subculture was not significant ($P > .05$). Meanwhile, the level of MDA of the T4-23 subculture may be an error of determination (Table 1). The hyphal growth of subcultures at the senescent phase was limited, and thus their MDA level may show no difference with that of subcultures at late juvenile phase as T6-16 manifested (Table 2), rather than significantly smaller than that of the T4-21 and T4-18 subcultures (Table 1). However, the difference of lipid peroxidations of the 10th, 12th, 14th, and 16th subcultures of *M. sextelata* T6 was not strong (Table 2). The detailed MDA variations in *M. importuna* and *M. sextelata* may reflect the different growth behavior and control mechanism of aging in two morels. The underlying mechanism needs further study. The results also implied that lipid peroxidation may be used as the index of morel aging cultures. Nevertheless, the threshold for aging cultures of different cultivated varieties was difficult to define.

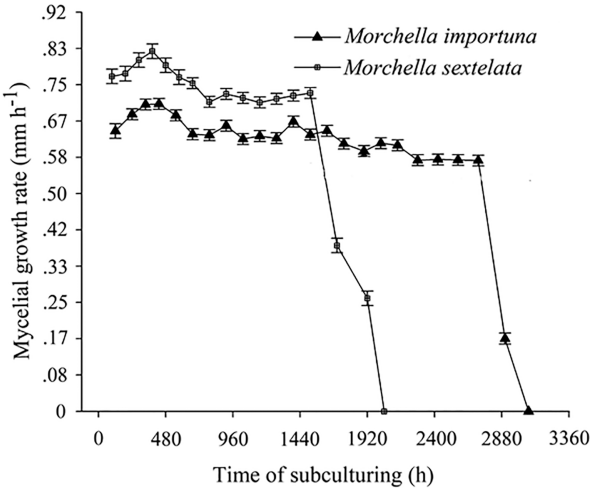

FIG. 1: Growth curve of *Morchella importuna* T4 and *M. sextelata* T6 successively subcultured on CYM plates

TABLE 1: Lipid Peroxidation (± SD), Mycelial Growth Rate (± SD), Biomass (± SD), and Average Number of Sclerotia Per Plate of Different Subcultures of *Morchella importuna* T4

Subculture no.	0	3	6	9	12	15	18	21	23
Time of subculturing (h)	0	336	672	1032	1392	1752	2136	2568	2904
Lipid peroxidation (mmol MDA/g fresh mycelia)	3.523 ± 0.056[a]	3.747 ± 0.067[a]	5.719 ± 0.081[b]	8.144 ± 0.124[c]	8.777 ± 0.126[d]	9.807 ± 0.152[e]	10.87 ± 0.212[f]	11.42 ± 0.191[g]	10.32 ± 0.202[h]
Mycelial growth rate (mm/h)	0.589 ± 0.002[a]	0.587 ± 0.004[a]	0.592 ± 0.006[a]	0.591 ± 0.001[a]	0.586 ± 0.006[a]	0.596 ± 0.004[a]	0.598 ± 0.003[a]	0.570 ± 0.006[b]	0.561 ± 0.003[b]
Biomass (g/d in fresh weight)	0.142 ± 0.002[a]	0.137 ± 0.004[a]	0.138 ± 0.003[a]	0.134 ± 0.004[a]	0.135 ± 0.003[a]	0.136 ± 0.003[a]	0.134 ± 0.003[a]	0.131 ± 0.002[b]	0.131 ± 0.004[b]
Average sclerotia per plate	> 101	> 101	> 101	51 ~ 100	51 ~ 100	51 ~ 100	51 ~ 100	< 50	< 50

Means within a row labeled with the same letter are not significantly different (Tukey HSD, $P \geq .05$).

TABLE 2: Lipid Peroxidation (± SD), Mycelial Growth Rate (± SD), Biomass (± SD), and Average Number of Sclerotia Per Plate of Different Subcultures of *Morchella sextelata* T6

Subculture no.	0	2	4	6	8	10	12	14	16
Time of subculturing (h)	0	192	384	576	792	1032	1272	1512	1920
Lipid peroxidation (mmol MDA/g fresh mycelia)	2.268 ± 0.060[a]	6.274 ± 0.084[b]	10.34 ± 0.090[c]	11.26 ± 0.141[c,d]	11.26 ± 0.122[d]	14.41 ± 0.132[e]	13.30 ± 0.154[e]	13.33 ± 0.108[e]	12.68 ± 0.120[e]
Mycelial growth rate (mm/h)	0.565 ± 0.003[a]	0.558 ± 0.004[a]	0.580 ± 0.005[a]	0.576 ± 0.002[a]	0.562 ± 0.004[a]	0.596 ± 0.004[a]	0.552 ± 0.006[a]	0.213 ± 0.003[b]	0.166 ± 0.002[b]
Biomass (g/d in fresh weight)	0.117 ± 0.002[a]	0.112 ± 0.003[a]	0.115 ± 0.003[a]	0.096 ± 0.004[a]	0.120 ± 0.002[a]	0.098 ± 0.004[a]	0.100 ± 0.003[a]	0.006 ± 0.001[b]	0.001[b]
Average sclerotium per plate	51 ~ 100	51 ~ 100	51 ~ 100	51 ~ 100	< 50	< 50	< 50	0	0

Means within a row labeled with the same letter are not significantly different (Tukey HSD, $P \geq .05$).

C. Cultural Characteristics of Subcultures

The cultural characteristics of healthy strains of *M. importuna* and *M. sextelata* on CYM plate were similar. Cultured at 24°C in the dark, the hyphae grew evenly at the rate of 0.58 ~ 0.86 mm/h (Fig. 1). The mycelia almost covered the whole plate (9 cm in diameter) in 3 ~ 4 d. Small white to light yellow sclerotial initials formed from highly proliferating interwoven hyphae after 5 ~ 7 d of incubation. Then the sclerotia matured after several days of development with thickening of sclerotial edges into convexities. Pigment secretion resulted in deeper color of the colony and sclerotia (Fig. 2).[19] Furthermore, the sclerotia of *M. importuna* T4 formed on each CYM plate (> 101) (Fig. 2) was more than those of *M. sextelata* T6 (51 ~ 100) (Tables 1 and 2). The changing trends of mycelial growth rate and biomass in subcultures of both *Morchella* spp. were similar; those of the last two subcultures (T4-21 and T4-23 of *M. importuna*, and T6-14 and T6-16 of *M. sextelata*) were significantly lower than other subcultures (Tables 1 and 2). In addition, the number of sclerotia formed on CYM plates tended to decrease in both morels (Fig. 2) and can be roughly categorized into three groups, i.e., > 101 (T4-0, T4-3 and T4-6), 51 ~ 100 (T4-9, T4-12, T4-15 and T4-18), and < 50 (T4-21 and T4-23) in *M. importuna* (Table 1), and 51 ~ 100 (T6-0, T6-2, T6-4 and T6-6), < 50 (T6-8, T6-10 and T6-12), and 0 (T6-14 and T6-16) in *M. sextelata* (Table 2). In addition, pigmentation of subcultures tended to increase with the rise of subculturing times (Fig. 2), which was also found in senesced strains of *Podospora anserina*.[37] The results indicated that the mycelial growth rate, biomass, and number of sclerotium may be used as the signs of seriously aging cultures. In other words, the degree of aging in most cultures of *M. importuna* and *M. sextelata* was difficult to estimate by cultural characteristics.

D. Cultivated Characteristics of Subcultures

Outdoor planting experiments were conducted in 2016–2017. Compared with other isolates, the hyphae grew slower, the pigmentation was denser, and fewer sclerotium were produced in the process of spawn production of subcultures at the senescent phase (T6-16 and T4-23). The cultivation of T6-16 and T4-23 was ultimately abandoned because the mycelia cannot grow well in the medium of final spawn. The results of other isolates suggested that the yield of both morels decreased with the rise of subculturing times (Figs. 3 and 4). Regression analysis showed a significantly negative correlation between time of subculturing and yield in *M. importuna* T4 (r = –0.882, P = 0.002) and *M. sextelata* T6 (r = –0.951, P

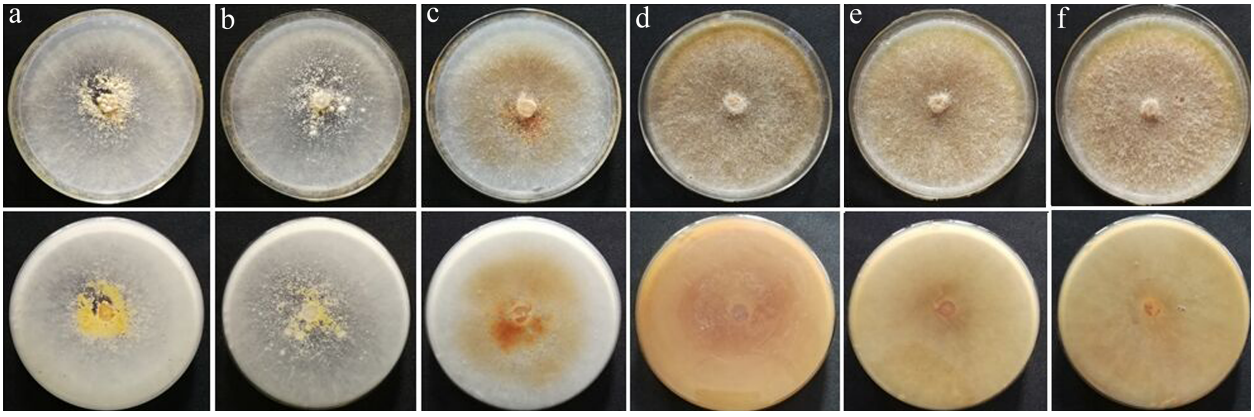

FIG. 2: Colony morphology of the original strain of *Morchella importuna* T4 (T4-0, a) and the 3rd (T4-3, b), 6th (T4-6, c), 9th (T4-9, d), 12th (T4-12, e) and 15th (T4-15, f) subculture cultured on CYM plates at 24°C in dark place for 14 d. The lower row was morphology of the reverse side of the corresponding plate in the upper row.

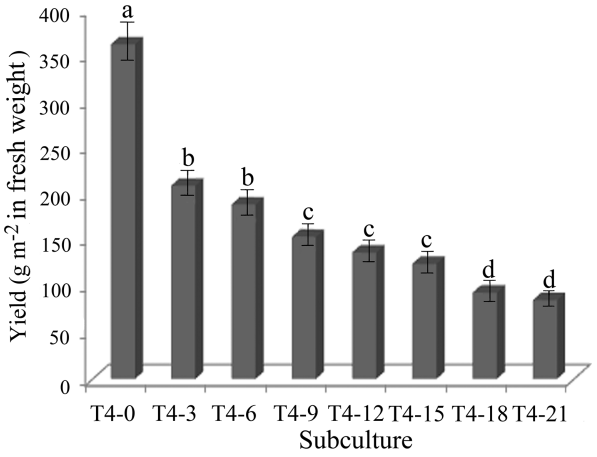

FIG. 3: Yield of subcultures of *Morchella importuna* T4. Data are presented as mean ± SD (n = 3). Different letters appearing above the columns indicate significant difference ($P < .05$).

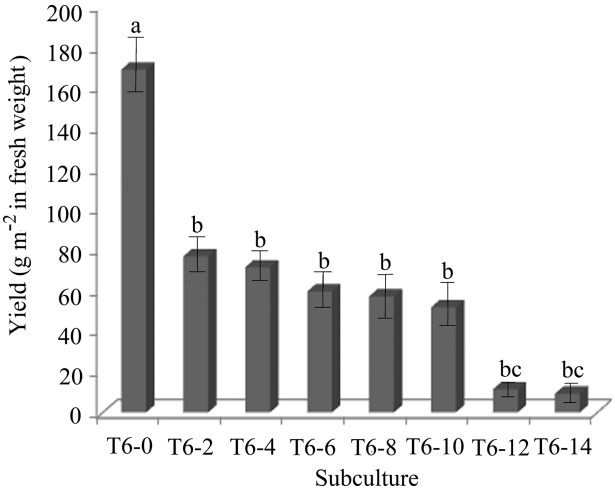

FIG. 4: Yield of subcultures of *Morchella sextelata* T6. Data are presented as mean ± SD. (n = 3). Different letters appearing above the columns indicate significant difference ($P < .05$).

= .000). Moreover, the yield of original strains (T4-0 and T6-0) was significantly higher than that of the subcultures ($P < .05$), which highlighted the application of healthy strains in morel artificial cultivation. However, the detailed yield categories of subcultures of *M. importuna* T4 and *M. sextelata* T6 were different. The yield of subcultures of *M. importuna* T4 can be categorized into three groups ($P < .05$), i.e., T4-3 and T4-6; T4-9, T4-12, and T4-15; and T4-18 and T4-21 (Fig. 3), whereas that of M. sextelata T6 was categorized into two groups: T6-2, T6-4, T6-6, T6-8, and T6-10 ($P > .05$) as well as T6-12 and T6-14 ($.01 < P < .05$) (Fig. 4). The results demonstrated that the effect of aging (subculturing) on yield of *M. sextelata* T6 was more serious than that of *M. importuna* T4. The yield of subcultures of *M. importuna* T4 decreased gradually (Fig. 3). However, in *M. sextelata* T6, the yield of subcultures was significantly

lower than that of T6-0, although not seriously different among the subcultures (Fig. 4). The excessive response of yield of *M. sextelata* T6 to aging (subculturing) may put forward higher request for application of the variety in artificial cultivation. In addition, the yield of *M. sextelata* T6-0 was lower than that of *M. importuna* T4-0 (Figs. 3 and 4), which was consistent with the overall performance of the strains in the production season. Finally, no significant difference was observed in other cultivated characteristics, e.g., time and quantity of mitospore appearance, time of fruiting, shape and size of ascocarps, and so on.

IV. DISCUSSION

A. Cultural and Cultivated Characteristics Associated with *Morchella* Aging Were Studied

In this study, systemic senescence of the cultivated strains of *M. importuna* T4 and *M. sextelata* T6 was achieved through successive subculturing, and then the cultural and cultivated characteristics associated with aging were determined. The results suggested that sclerotial formation and fructification of the subcultures of *M. importuna* and *M. sextelata* tend to weaken with the rise of subculturing times (Tables 1 and 2; Figs. 2–4). Successive subculturing has been used in animal, human, and filamentous fungal cells for study of replicative senescence *in vitro*.[32,38] The study of strain stability through successive subculturing is essential for selecting the best strain for commercial purposes.[31] Correspondingly, this work is beneficial in recognizing and avoiding the use of aging spawn in morel cultivation as well as promoting spawn production of *Morchella* mushrooms.

B. Aging Study Was Instructive for *Morchella* Production

Morel researchers and farmers have been frustrated at the "uncertainty" of morel farming. The underlying reasons may be complicated and spawn aging may be involved. Our study found that the degree of aging in most morel cultures was difficult to estimate by cultural characteristics, which implicated that the application of aging cultures in morel cultivation may be common and mainly attributed to the "uncertainty" of morel farming. Application of scientific technical measures, e.g., eliminating subculturing times, incubating at low temperature, preserving spawn properly, and rejuvenating the isolates without delay, may effectively alleviate the damage of spawn aging. In addition, the study found that *M. sextelata* T6 was apt to senesce compared with *M. importuna* T4. The result was in accordance with morel production. *M. importuna* T4 has been widely applied in commercial cultivation for more than 4 years with ideal performances. In contrast, T6 has been gradually replaced with T-13 of *M. sextelata* after 3 years of commercial application because of spawn aging. The cultures of T6 preserved for a long time and subcultured many times should be used with caution in production. Furthermore, the cultivation temperature for starting culture, primary spawn, and culture spawn should be low (below 22°C) to effectively reduce spawn aging. Some strains of *M. sextelata* showed stronger resistance to microtherm, drought, and rough management measures than those of *M. importuna* and thus have been widely popularized in North China. Therefore, sheltering from spawn aging, especially for *M. sextelata*, will be beneficial for stable and long-term development of morel farming.

V. CONCLUSIONS

The prominent problem of "uncertainty" has been frustrating morel farming since the commercial production of *Morchella* mushrooms was realized in China. Spawn aging may be the main reason for "uncertainty." Unfortunately, morel aging has not been studied systematically. Using the cultivated strains of *M.*

sextelata T6 and *M. importuna* T4, aging was first achieved by successive subculturing, and then the effects of aging on morel culture and cultivation were studied. The culture experiments suggested that the mycelial growth rate, biomass, and number of sclerotium may be used as signs of seriously aging cultures. Outdoor planting experiments demonstrated that yield decreased with the rise of time of subculturing. The seriously aging cultures with apparently slow mycelial growth, denser pigmentation, less sclerotial formation, and low or null yield are not suitable for production. Moreover, *M. sextelata* T6 was apt to senesce in comparison with *M. importuna* T4. The results were consistent with and instructive for *Morchella* cultivation. The application of aging cultures in *Morchella* cultivation may be common and mainly attributed to the "uncertainty" of morel farming. Further study of aging mechanism will promote the stable development of *Morchella* farming.

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