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Fruit-body production and mycelial growth of *Tephrocybe tesquorum* in urea-treated forest soil

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Tephrocybe tesquorum **is an ammonia fungus that forms reproductive structures successively on the forest floor after treatment of the soil with nitrogenous materials such as aqua ammonia and urea. Forest soil was treated with urea at the rates of 0, 5, 10 and 20 mg/g fresh soil for 5 d in the laboratory, then sterilized by gamma-irradiation. Vegetative hyphae of** ***T. tesquorum*** **were inoculated into the sterilized soil, and the number and weight of fruit-bodies formed and the length of vegetative hyphae were measured for 20 d after the inoculation. Only in the urea-treated soil did this fungus produce vegetative hyphae and fruit-bodies. Fruiting started 4 to 6 d after inoculation. The weight of fruit-bodies and the length of vegetative hyphae increased with the increase in the amount of urea added. These results indicate that** ***T. tesquorum*** **develops vegetative hyphae and fruit-bodies when ammonium concentration is high in soil.**

Key Words——ammonia fungi; fluorescent dyes; fruiting; mycelial growth; sterilized soil.

Macrofungal species play an important role in nutrient dynamics in forest ecosystems. Some of these species are saprotrophs with high enzymatic abilities and others are symbionts forming mycorrhizal associations with trees. The ecology of macrofungi has been well documented, including information on their distribution, seasonal abundance and substratum preference (e.g., Dix and Webster, 1995). Physiological characteristics and host specificity in mycorrhizal formation are well understood for some fungi in the laboratory. However, the relationship between mycelial development and fruit-body appearance is known only for *Agaricus bisporus* (Lange) Singer, *Schizophyllum commune* Fr. and some coprophilous species (Wessels, 1965; Wood, 1976; Wood and Cooke, 1987; Safar and Cooke, 1988a).

Tephrocybe tesquorum (Fr.) M. M. Moser (syn. *Lyophyllum tylicolor* (Fr.: Fr.) M. Lange & Sivertsen), a small agaric species, forms fruit-bodies exclusively after the addition of some nitrogenous materials such as urea and aqua ammonia to forest soil (Sagara, 1975). Under natural conditions, this fungus fruited on forest soil amended with human urine or in which the corpse of a cat or dog had decomposed (Sagara, 1975). It also appeared 'on what apparently were the very decayed remains of some fleshy fungus' (Smith, 1941, under the name *Collybia olympiana* A. H. Sm.). This species grows well on various nitrogen sources, such as ammonium salts, amino acids, urea and bovine serum albumin (Suzuki, 1989; Yamanaka, 1999). It also decomposes cellulose, lignin, chitin, protein and lipid (Enokibara et al, 1993; Yamanaka, 1995; Soponsathien, 1998). These results indicate that *T. tesquorum* develops mycelia and fruit-bodies in forest litter in the presence of high concentrations of ammonium nitrogen and plays an important role in decomposition of waste materials. Formation of fruit-bodies and basidia on a small amount of mycelia (Yamanaka and Sagara, 1990; Yamanaka, 1994), however, shows that this species may sporulate without a large amount of mycelia in forest litter.

In the present study, a culture of *T. tesquorum* was inoculated on urea-treated and gamma-sterilized forest soil and fruit-bodies were obtained. Mycelial biomass was estimated by measurement of hyphal length on membrane filters (Sundman and Sivelä, 1978) using the fluorescent dyes, fluorescein diacetate (Söderström, 1977) and fluorescent brightener 28 (West, 1988) to distinguish living hyphae from dead ones. This method is more rapid and convenient than the estimation of chitin and ergosterol content. In this way, the changes in mycelial biomass during fruit-body development and the effects of urea treatment on the production of vegetative hyphae and fruit-bodies were examined.

Materials and Methods

Organism Fruit-bodies of *T. tesquorum* were collected from forest soil treated with 700 g of urea/m^2 on 19 Apr 1991 in *Pinus densiflora* Zucc. & Sieb. forest at Kamigamo Experimental Forest Station of Kyoto University, Japan. Pure cultures were obtained from germinating basidiospores collected from one of the fruit-bodies and maintained on glucose-yeast extract agar medium (1% (w/v) glucose, 0.2% yeast extract (Difco) and 1.5% agar)

Soil preparation In Dec. 1991, soil was collected from the organic soil layer (A_0 layer) in the same forest and

transported to the laboratory, where portions of 200 g were dispensed into plastic pots (14 cm in inner diam and 11 cm in depth). Urea was added to the soil at the rate of 5, 10 and 20 mg/g fresh soil, followed by 200 ml of distilled water. Soil with only distilled water added served as a control. The urea-treated soils, hereafter referred to as 5, 10 and 20 mg urea soil, respectively, were left in the laboratory for 5 d to allow the added urea to decompose to ammonia, then oven-dried at 40°C. The dried soil was passed through a sieve (<3 mm) and placed into plastic bags, where it was sterilized by gamma-irradiation (5 Mrad) and stored until required. The pH of these treated soils was measured with a glass electrode after suspending 1 g of dry soil in 20 ml of distilled water. The concentrations of ammonium and nitrate in the soils were determined colorimetrically by an indophenol-blue method and a modified Griess-Ilosvay method, respectively (Keeney and Nelson, 1982). The sterilized soil (1 g of dry weight) was placed into a 15-ml polycarbonate cup (23 mm in inner diam and 47 mm in depth) with 4 holes at the bottom to drain off excess water. The cup was then put into a 50-ml screw vial, the outside of which was covered with black vinyl tape. The soil was rehydrated with 3 ml of sterilized water and placed in the laboratory 1 d before inoculation.

Inoculation and incubation A pure culture of *T. tesquorum* was incubated for 2 wk at 20°C in liquid medium containing 1% (w/v) glucose and 0.2% yeast extract, then stirred at 10000 rpm for 30 s using a Cartridge Mill (Ikeda Scientific). The hyphal pellet was collected by centrifugation at 1000 rpm for 5 min, and the supernatant solution was discarded. The pellet was washed with sterilized water three times and resuspended in sterilized water. This hyphal suspension was used as an inoculum. One ml of the suspension containing ca. 53 μg of dry hyphae was added to the soil in the cup. The inoculated soil in the vial was incubated at 20°C with a light intensity of 400–700 lux at the top of the vials. Soil was watered with 4 ml of sterilized water 5, 10, and 15 d after inoculation.

Sampling Fruit-body production and mycelial biomass in soil were examined 2, 4, 6, 8, 10, 15 and 20 d after inoculation. On each sampling day, five replicates for each soil treatment were sampled. The structures with pilei were counted as fruit-bodies and their fresh weight and dry weight after oven-drying at 105°C for 5 h were determined.

Staining of hyphae with FDA or FB and measurement of hyphal length The length of hyphae in the soil was measured after staining with fluorescein diacetate (FDA; Sigma) or fluorescent brightener 28 (FB; Sigma). After removing fruit-bodies, the soil (1 g of dry weight) was suspended in 95 ml phosphate buffer (60 mM, pH 7.6, filtered through a 0.22-μm pore size membrane filter) and stirred for 30 s at 10000 rpm. This suspension was then diluted to 10^{-1}. To 1 ml of the diluted suspension, 1 ml FDA (20 μg/ml the buffer) and 1 ml of the filtered buffer were added. After 10 min, the suspension was filtered through a black membrane filter (0.8-μm pore size, Advantec Toyo). The filter was washed twice with 10 ml of the buffer, then placed on a slide glass. A coverslip with a drop of the phosphate buffer was placed on the filter. The hyphae on the filter were observed under an epifluorescent microscope at ×40 magnification. Fields were photographed along a vertical transect through the center of the filter, and the hyphae were measured on the photograph with a map measurer.

The soil suspension stirred for 30 s at 10000 rpm for the determination of FDA-stainable hyphae was also used for measuring FB-stainable hyphae by subsequent blending at 19500 rpm for 2 min. This suspension was diluted to 10^{-2}. An aliquot (1 ml) was removed from the diluted suspension and mixed with 0.1 ml of 0.1% (w/v) FB and 2 ml of the filtered buffer. Ten min later, the suspension was filtered through a 0.22-μm pore size polycarbonate membrane filter (Nucleopore) which had been previously stained with Sudan B (Merck) as described by Zimmermann et al. (1978). The filter with soil sample was washed as described in the above paragraph, then attached to a slide glass with a drop of non-fluorescent immersion oil (Type DF, Cargille). A coverslip was put in place after the addition of another drop of the immersion oil onto the filter. The filter was observed by epifluorescent microscopy at ×100 magnification. The hyphal length was estimated by the grid intersection method (Olson, 1950; West, 1988). For each membrane filter 20 fields of view were counted.

Statistical analysis Kruskal-Wallis test was used to determine the significance of mean differences between urea treatments and between days after inoculation (Ishii, 1975). Soil chemical characteristics were not statistically analyzed because only two samples of each urea treatment were investigated. The experiments for the measurement of fruit-bodies and vegetative hyphae were repeated twice with similar results.

Results

Soil characteristics The ammonium concentration and pH value of the sterilized soil increased with the amount of urea added, but the nitrate concentration was the same in all urea-treated soils (Table 1). Even in the soil without urea treatment, the ammonium concentration was higher than that in the soil sampled in the field (Yamanaka, 1995), possibly due to the release of ammonium from soil organic matter during oven-drying.

Appearance of fruit-bodies Fruiting started 4 d after inoculation in 10 and 20 mg urea soil, and 6 d in 5 mg urea soil (Fig. 1). No fruit-bodies appeared in the soil without urea added (Figs. 1, 2). The number of fruit-bodies was generally the same in all urea treated soils, but the fresh weight and dry weight of fruit-bodies were significantly heavier in 10 and 20 mg urea soil than in 5 mg urea soil. There were no significant differences in the fresh or dry weight of fruit-bodies between 10 and 20 mg urea soil throughout the experimental period. The ratio of dry to fresh weight of fruit-bodies in 10 and 20 mg urea soil decreased with time after inoculation (Table 2).

Length of FDA- or FB-stainable hyphae FDA stains living hyphae by reaction with protoplasm of hyphal cells

Table 1. The pH value and concentration of NH_4^+-N and NO_3^--N in sterilized forest soil treated with various amounts of urea. Values are the means from two replicates.

Amount of urea added[a]	pH	NH_4^+-N[b]	NO_3^--N[c]
0	3.8	0.17	8.2
5	5.5	4.9	5.9
10	6.3	7.8	5.7
20	6.8	10.5	6.5

[a] mg/g fresh soil.
[b] mgN/g dry soil.
[c] μgN/g dry soil.

and FB stains both dead and living hyphae, impacting fluorescence to the cell walls and septa of hyphae (Fig. 3). Initially the FDA-stainable hyphae increased sharply, reaching a maximum 4 or 6 d after inoculation, and they declined thereafter (Fig. 4a). The length of the hyphae stained with FDA increased in parallel with the amount of urea added initially. In the control cultures, no reactive hyphae were detected. Fifteen and 20 d after inoculation, large bacterial populations were observed in many of the urea-treated soil samples.

Uninoculated soil contained a certain amount of FB-stainable hyphae. In all treated soils, the estimated length of hyphae sharply increased during the initial 4 d after inoculation and then remained unchanged (Fig. 4b). The highest yield of stained hyphae was obtained in 20 mg urea soil. In the FB-stained samples, bacteria were not recognized.

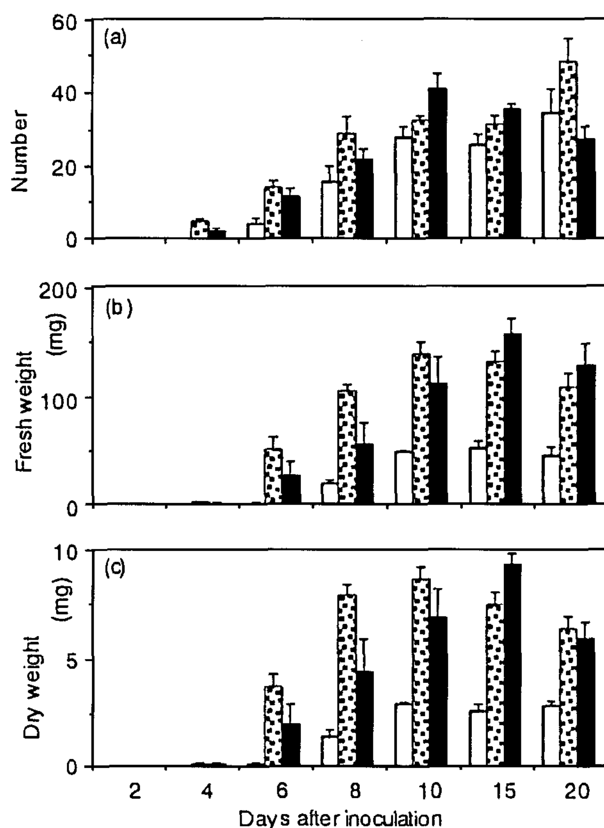

Fig. 1. Number (a), and fresh (b) and dry (c) weight of fruit-bodies of *T. tesquorum* developed on the soil to which 5 (□), 10 (▩), and 20 (■) mg urea per g fresh soil was added. Values are the means with standard errors from five replicates. No fruit-bodies were formed in the soil without added urea.

Table 2. The ratio of dry to fresh weight (%) of fruit-bodies of *T. tesquorum*. Values are the means of five replicates. Values followed by different letters within columns are significantly different at $P<0.05$ according to Kruskal-Wallis test.

Days after inoculation	Amount of urea added		
	5[a]	10	20
4	—[b]	—	—
6	—	7.6a	9.1a
8	7.9a	7.7a	8.1ab
10	5.9a	6.5ab	6.4bc
15	5.1a	5.6b	6.0bc
20	7.5a	6.0b	4.8c

[a] mg/g fresh soil.
[b] No data were obtained, because fresh weight of fruit bodies was too small to measure its dry weight or no fuit-bodies were formed.

Discussion

In studies on coprophilous fungi, a 'coprome' (Wood and Cooke, 1984), a semi-natural resource unit which was made from a laboratory-fed rabbit and sterilized by gamma-irradiation, has been employed for clarifying antagonisms among fungi (Safar and Cooke, 1988a) and between fungi and bacteria (Safar and Cooke, 1988b), and for examining changes in mycelial biomass in relation to sporulation (Wood and Cooke, 1987). In the present study, the soil for culture of *T. tesquorum* was prepared as a homogenous substrate after sieving, and the amount of soil used was smaller than that in nature. *Tephrocybe tesquorum* grows under a physically and chemically heterogeneous environment on the forest floor, but this is not a serious problem so long as comparison was made between the amount of urea applied and the production of fruit-bodies and vegetative hyphae. Colonization and fructification of this fungus in a small amount of field soil reported by Yamanaka and Sagara (1990) also show that the present results in a microcosm study may reflect the results obtained in the field.

In the unsterilized soil, this fungus fruits 17 d after urea treatment in the laboratory (Sagara, 1976). In the present study this fungus formed fruit-bodies on the sterilized soil 4 d after inoculation (Fig. 1), indicating that the growth of *T. tesquorum* is affected by competitive interactions among fungi or between fungi and other soil organisms. In the case of coprophilous fungi, some ascomycetes and *Coprinus* species fruited on sterilized dung earlier than on fresh dung (Harper and Webster, 1964). In the studies using copromes, fruiting of some ascomycetous coprophilous fungi was reduced in the

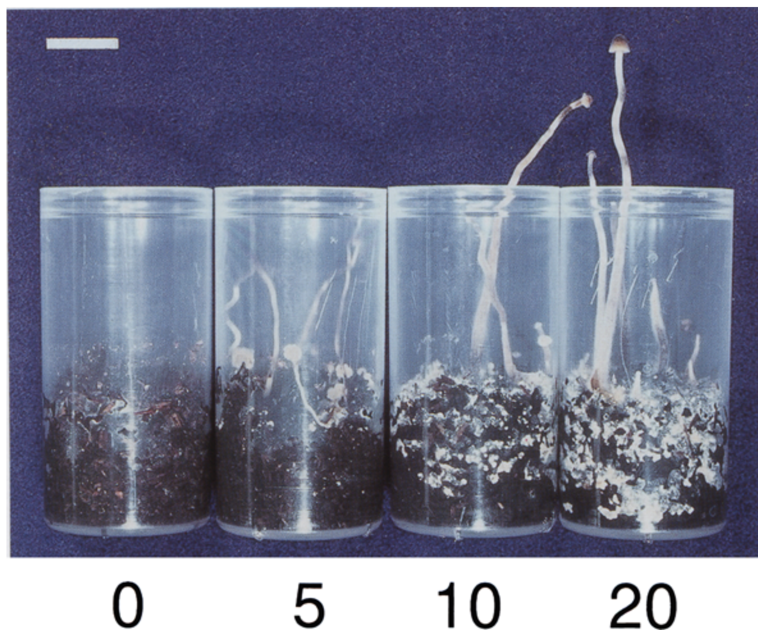

Fig. 2. Fruit-bodies of *T. tesquorum* developed on the soil to which 0, 5, 10 and 20 mg urea per g fresh soil was added. The cups from left to right represent 0, 5, 10 and 20 mg urea per g fresh soil. Photographs were taken 15 d after inoculation. Note that this fungus grew and formed fruit-bodies only on urea-treated soil. Scale bar = 10 mm.

presence of one or two other fungi (Safar and Cooke, 1988a, b).

Tephrocybe tesquorum formed fruit-bodies earlier in 10 and 20 mg urea soil than in 5 mg urea soil (Fig. 1). The length of vegetative hyphae in 10 and 20 mg urea soil was longer than that in 5 mg urea soil (Fig. 4), and thus these results were consistent with Buller's view (1931) that a considerable amount of mycelia is required for the production of fruit-bodies. However, fruit-bodies were formed on the 6th day after inoculation in 5 mg urea

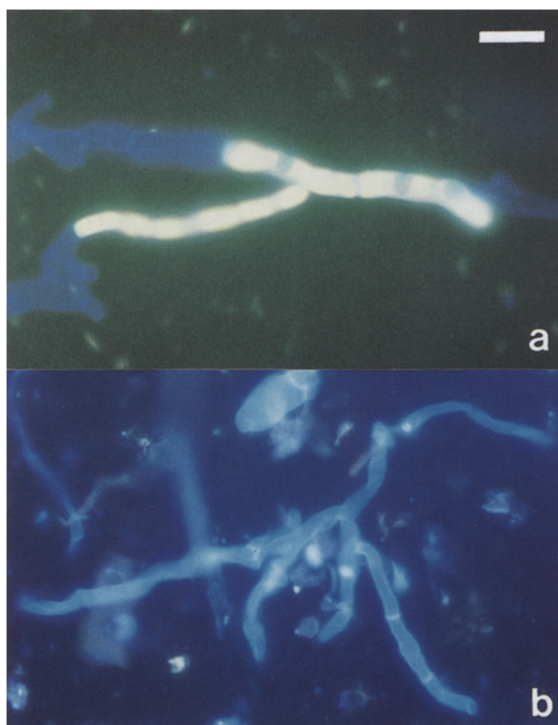

Fig. 3. Vegetative hyphae of *T. tesquorum* stained with fluorescein diacetate (a), showing protoplasm of hyphal cell; and fluorescent brightener 28 (b), which reacted with cell wall and septa of hyphae. Scale bar = 20 μm.

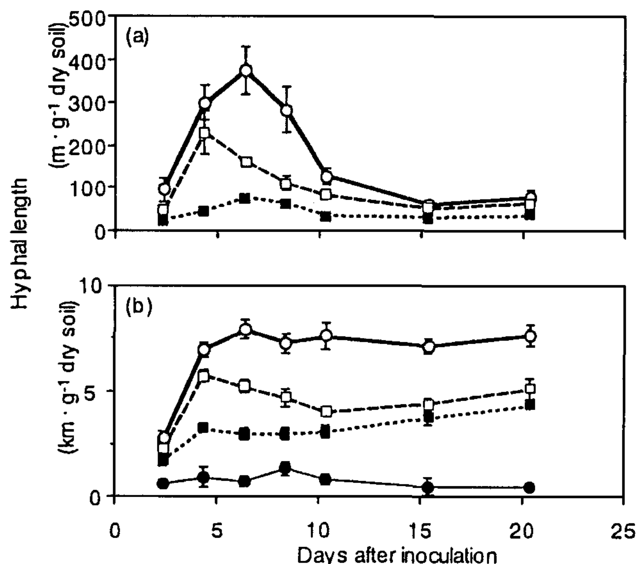

Fig. 4. Hyphal length of *T. tesquorum* in soil to which 5, 10, and 20 mg urea per g fresh soil was added. The hyphal length was estimated after staining with fluorescein diacetate (a) or fluorescent brightener 28 (b). ●, control (untreated); ■, 5 mg; □, 10 mg; ○, 20 mg. Values were the means with standard errors from five replicates. No hyphae were detected in control soil after staining with fluorescein diacetate.

soil (Fig. 1), although the amount of vegetative hyphae was smaller than that on the 4th day in 10 and 20 mg urea soil (Fig. 4). These results, therefore, suggest that during a certain period of vegetative growth some specific fruiting substance is formed or medium conditions favorable for fruiting are established, as stated by Madelin (1956) in the study of *Coprinus lagopus* Fr.

Fruit-body primordia of *T. tesquorum* were formed during the period of active mycelial growth indicated by the increase in length of FDA-stainable hyphae (Figs. 1, 4a). Suzuki (1989) also mentioned that basidiocarps of *T. tesquorum* were produced during the phase of linear growth. Wessels (1965) reported that primordium initials of *S. commune* were formed during colony growth, whereas Schwalb (1971) observed that fruiting and vegetative growth of mycelium in *S. commune* might show a quantitatively inverse relation. Wood (1976) found the formation of fruit-body primordia of *A. bisporus* after the cessation of vegetative growth, and considered that the process is similar to catabolite repression because the addition of carbohydrates to the agar medium repressed primordium formation of *A. bisporus* but supported good vegetative growth. The inhibition of fruit-body formation by a higher concentration of glucose in the culture medium has been observed not only in *A. bisporus*, but also in *Coprinus macrorhizus* Rea f. *microsporus* Hongo, *Asterophora lycoperdoides* (Bull.) Ditmar and *T. tesquorum* (Uno and Ishikawa, 1974; McMeekin, 1991; Yamanaka, 1994).

The dry weight of fruit-bodies was correlated with the length of vegetative hyphae (Figs. 1, 4). A similar parallelism between fruit-body production and the amount of vegetative hyphae was also observed in *A. bisporus* (Wood, 1976). Furthermore, in the present study, the length of vegetative hyphae and the dry weight of fruit-bodies of *T. tesquorum* increased with the concentration of urea added to the soil, i.e., the concentration of ammonium in the treated soil (Table 1; Fig. 1). The size and weight of fruit-body of *Hebeloma vinosophyllum* Hongo, another species belonging to the same chemoecological fungal group as *T. tesquorum*, increased with the amount of urea added (Sagara, 1976). *In vitro* studies using some species of this fungal group show that spore germination, mycelial growth and fruiting were stimulated by ammonium ions (Suzuki, 1978, 1989; Morimoto et al., 1981; Suzuki et al., 1982; Yamanaka, 1999). *Tephrocybe tesquorum* also fruited on agar medium containing the extract from urea-treated forest litter (Yamanaka, 1994). All of these results indicated that ammonia is a key substance for the vegetative and reproductive growth of the ammonia fungi.

The ratio of dry to fresh weight of the fruit-bodies of *T. tesquorum* in 10 and 20 mg urea soil decreased with fruit-body maturation (Table 2). For example, the ratio in 20 mg urea soil was 9.1% on the 6th day after incubation and 4.8% on the 20th day after incubation. The ratio of dry to fresh weight of the fruit-bodies of *Coprinus congregatus* Bull. ex Fr. decreased during their maturation (Robert, 1977), but in *Agaricus campestris* L.: Fr. the ratio remained the same throughout the elongation period of the stipe (Bonner et al., 1956). Robert (1977) considered the preferential translocation of water into developing fruit-bodies to be a reason for the decrease in the ratio. Furthermore, Manachère (1970) suggested that cell components such as proteins and carbohydrates might be transferred into vegetative mycelia after the development of a fruit-body in *C. congregatus*. In the present study, the amounts of both FDA- and FB-stainable hyphae remained unchanged after fruit-bodies had developed at 10 d after inoculation (Fig. 4), indicating that the vegetative hyphae in soil were exhausted during fruiting and that no cell components were transferred to the vegetative hyphae from matured fruit-bodies of *T. tesquorum*.

The growth form of *T. tesquorum* in untreated soil remains unknown. In pure culture, this fungus produced vegetative hyphae at pH 3 and 4 (Yamanaka, unpub. data), which is the pH level of untreated forest soil (Yamanaka, 1995). This indicates that *T. tesquorum* can grow vegetatively in untreated forest soil. In the present study, however, neither FDA-stainable hyphae nor fruit-bodies were detected in the untreated soil (Figs. 1, 2, 4). In addition, this species did not grow well on the medium without nitrogen sources (Yamanaka, 1999). These results suggest that *T. tesquorum* could produce neither vegetative hyphae nor fruit-bodies on untreated forest litter, and that, under such conditions, this species might be present in the dormant form such as spores or hyphal fragments.

The present results show that *T. tesquorum* starts to develop vegetative hyphae after urea treatment and then to form fruit-bodies. *Tephrocybe tesquorum* is one of the early-phase species of the ammonia fungi that fruits ca. 1–3 mo after urea treatment (Sagara, 1975; Yamanaka, 1995). The developmental pattern of *T. tesquorum* after urea treatment should be observable in other early-phase species of the ammonia fungi, because these species possess similar characteristics of nitrogen nutrition, enzymatic activity, and pH range favorable for vegetative growth (Suzuki, 1989; Enokibara et al., 1993; Yamanaka, 1995, 1999; Soponsathien, 1998; Yamanaka, unpub. data).

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