Principles of Artificial Propagation of *Tricholoma conglobatum* (Vitt.) Sacc.

I. Germination of the Spores and the Cultural Characters of the Fungous Mycelium.

By

Y. Nisikado and K. Kimura. (179)

[November 10, 1941.]

Contents

I. Introduction.

Tricholoma conglobatum (Vitt.) Sacc. or Simedi as it is called in Japanese has been known for centuries as excellent edible mushroom. In Okayama district, higher prices are often paid to this mushroom than the most well-known Matsutake mushroom, *Armellaria Matsutake* Ito et Imai. In spite of this popularity, there has been but a very limited amount of work done. The present study is the first of the series on the principles of artificial propagation of *T. conglobatum* with investigations on the germination of spores and the cultural characters of the fungous mycelium. The authors are indebted to the Osaka Forestry Bureau and to Messrs. Kozi Hirata and Kiyu Yamauti, both formerly of the Institute, for performing a portion of the experiments and to Mr. T. Nakayama for the preparation of the English text. Thanks are also due to the Okayama and Saijo Forestry Stations, and Mr. Hikozaburo Seno of Okayama City for supplying the materials used in this investigation.

II. Morphology.

The mushroom normally appears in clusters with the stipes grouped together. In the early stage the pileus is nearly black, but it changes to deep gray when gills

are exposed. The expanded pileus is nearly flat at the top; the margin is involute but becomes reflexed on aging. The gills are white and may be either adnate or sinuate to the stipe. The stipe is white and bulbous — sometimes as much as 3 cm. in diameter — at the base and attenuate toward the top; the diameter at the attachment of the pileus is 0.7 to 1.0 cm. The flesh is compact and is white in both the pilus and the stipe. (Plates I - III). A very similar mushroom occurring in Kyoto district has been named by MATSUURA as *Tricholoma Sakurashimeji* MATSUURA but discussions on this mushroom will be reserved for other occasion.

The spores are hyaline, unicellular, almost spherical, thin walled, and the content is granular. They measure 5 - 7 μ in length; a mode of 100 measured spores was 6.0 μ, and the average was 5.87 $\pm$ 0.021 μ. (Plate IV, Fig. 6).

III. Germination of Spores.

Spores of *T. conglobatum* were dropped on the surface of hardened 3% malt extract agar medium in a Petri dish by suspending some fragments of a well developed pileus over the roof of the container. It was found that 24 hours of incubation at 24 °C., produced a good germination on some of the spores, but with others 3 to 7 days were required. For a complete germination, a period of 7 to 10 days was often required, but it depended much on the material. (Plate IV, Fig. 6 - 7).

1. *Relation of Temperature to the Germination of Spores.*

Spores were dropped on the surface of 3% malt extract agar medium contained in a Petri dish and were incubated for a definite period of time at various temperatures. The percentage of germination was calculated after observing the

Tabele I.

Relation of Temperature to the Germination of Spores of *Tricholoma conglobatum* (Vitt.) Sacc.

Results after 5 days of incubation.

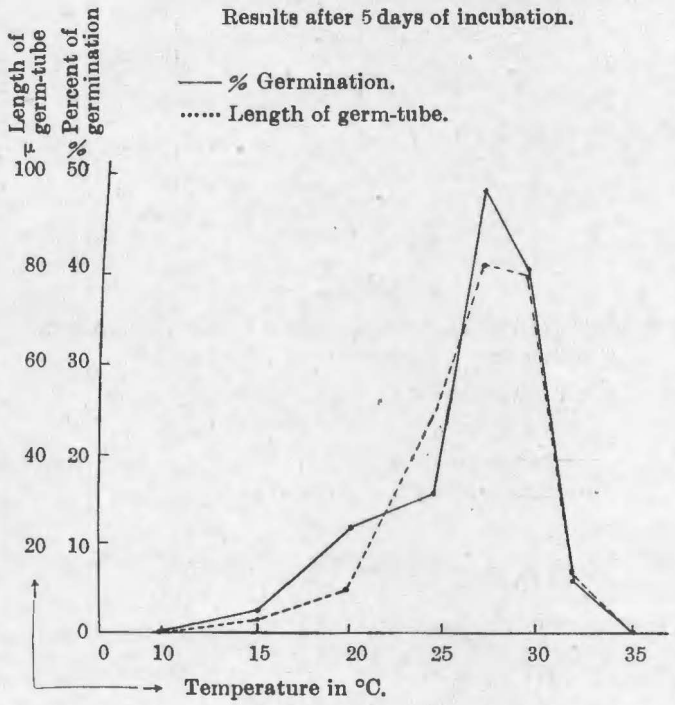
Temperature	Spore germination			Length of germ-tube	
	No. observed	No. germinated	% germination	Range	Average
5°C.	105	0	0	—	—
10°	208	0	0	—	—
15°	424	9	2.1	1 - 5 μ	3.20 μ
20°	497	56	11.3	1 - 110	8.70
24°	558	88	15.8	2 - 300	49.46
27°	344	168	48.8	5 - 400	83.50
29°	367	150	40.8	5 - 400	80.12
32°	346	18	5.2	1 - 20	12.00
35°	182	0	0	—	—

extent of germination, and also the length of the germ-tube was measured. To facilitate observation, a microscope with a movable stage was used. Mounts for observation were made by transferring cut pieces of agar with germinating spores on to glass slides, and sealing over with cover slips. Germinated and ungerminated spores were counted as they appeared in the field of the microscope; a total of 50 different fields were observed by shifting with the stage manipulators. The length of the germ-tube was measured directly with an ocular micrometer on all the spores appeared in each of the 25 different fields. The results of observations made after 5 days of incubation are shown in Table I and Figure I.

Figure I.

Relation of Temperature to the Germination of Spores of *Tricholoma conglobatum* (Vitt.) Sacc.

Results after 5 days of incubation.



According to Table I and Figure I, the spores germinated quite abundantly after 5 days in temperatures between 15° and 32 °C. The highest percentage of germination resulted in the temperature of 27 °C., and also the same temperature showed the longest germ-tubes. This was followed by 29 °C. and a marked decrease was noted at below 24 °C. and at 32 °C.

2. The Relation of Hydrogen-ion Concentration of the Culture Medium to the Germination of Spores.

3% malt extract agar medium with different hydrogen-ion concentrations was prepared by adding varying quantities of N/2 solutions of hydrochloric acid or

sodium hydroxide. The medium was poured in plates in Petri dishes, and the spores were allowed to drop on them. The Petri dishes were incubated at 27 °C. Table II and Figure II show the percentage of germination and the average

Table II.

Relation of Hydrogen-ion Concentration to the Germination of Spores of *Tricholoma conglobatum* (Vitt.) Sacc.

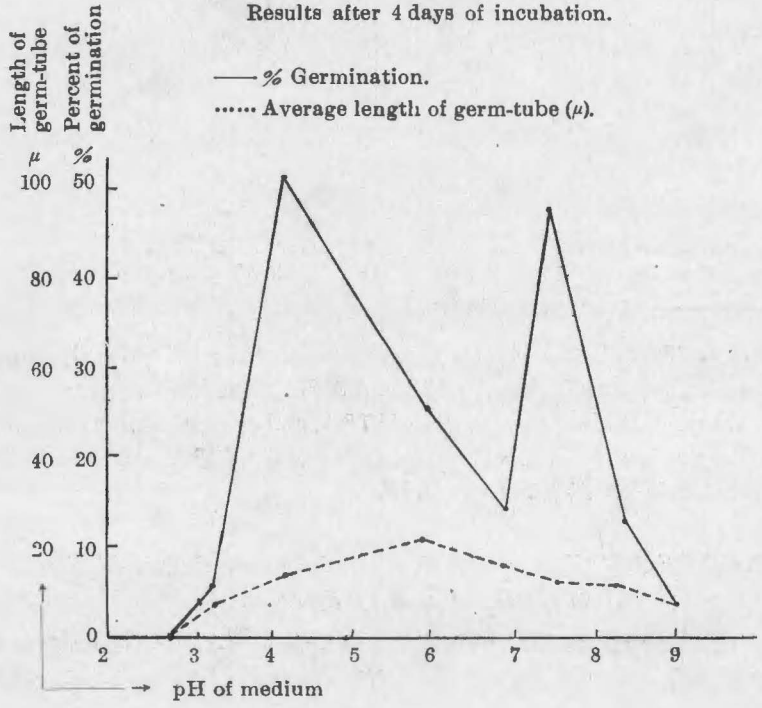
Results after 4 days of incubation.

pH of medium	Spore germination			Length of germ-tube	
	No. observed	No. germinated	% germination	Range	Average
9.0	377	10	2.7	1 – 3 μ	1.32 μ
8.4	252	29	11.5	1 – 7	2.37
7.4	468	227	48.5	1 – 9	2.51
6.9	384	54	12.3	1 – 22	3.51
5.8	348	91	26.2	1 – 25	5.22
4.1	297	212	71.4	1 – 120	2.90
3.2	240	12	5.0	1 – 2	1.63
2.7	110	0	0	—	—

Figure II.

Relation of Hydrogen-ion Concentration to the Germination of Spores of *Tricholoma conglobatum* (Vitt.) Sacc.

Results after 4 days of incubation.



length of the germ-tubes after 4 days of incubation. The table shows that the spores were able to germinate in a substratum that had a reaction between pH 3.2 and 9.0. The optimum pH for germination was 4.1, which was next followed by 7.4. On the other hand, the longest germ-tubes were noted at pH 5.8. The results showed that, in general, the spores are able to germinate best at the reactions that are either slightly acid (pH 4 to 6) or basic.

3. The Resistance of the Spores to Dry Heat.

The procedure for testing the resistance of spores to heat consisted in inserting spore-smeared cover-slips into an electrically heated oven, which was adjusted to various temperatures (50 to 150°C.). The cover slips were inserted from the little vent situated at the top of the oven and were set in sterilized Petri dishes. The introduction of cover-slips was carried out at 5 minutes interval for 30 minutes when the door of the oven was opened and the cover-slips removed for incubation of the spores for germination. This was accomplished by moistening the cover-slips with a few drops of sterile distilled water and placing them in moist chambers. The incubating temperature was held at 24 °C. Observations were made at the end of 3 and 5 days. The extent of germination was expressed by +, − and ± signs, indicating positive, negative, and very low germinations, respectively. In ±, most of the fields showed no germination at all.

Table III.

Resistance of Spores of *Tricholoma conglobatum* to Dry Heat.
Experimented on November 5, 1936.

Time of treatment	Temperature of treatment										
	50°C	60C°	70°C	80°C	90°C	100°C	110°C	120°C	130°C	140°C	150° C
5 min.	±	±	±	±	±	±	−	−	−	−	−
10 „	±	±	±	±	±	±	−	−	−	−	−
15 „	±	±	±	±	±	−	−	−	−	−	−
20 „	±	±	±	±	±	−	−	−	−	−	−
25 „	±	±	±	±	±	−	−	−	−	−	−
30 „	±	±	±	−	−	±	−	−	−	−	−

The results of Table III indicate that the spores are not killed by a temperature of 100 °C. when the exposure was about 10 minutes. Consequently it appears from this that the spores hidden in soil are able to survive the heat to some extent as the surface of the soil is burnt over with rice straw.

IV. Cultural Characters of the Mycelium.

When the germinated spores were transferred to test-tube slants of potato decoction medium, they form dense, white mycelial growth after 7 to 10 days.

A portion of this mecelium was employed in the studies on the relation of the fungus growth to temperature, hydrogen-ion concentration, type of culture medium, and sugar content of the medium.

Table IV.

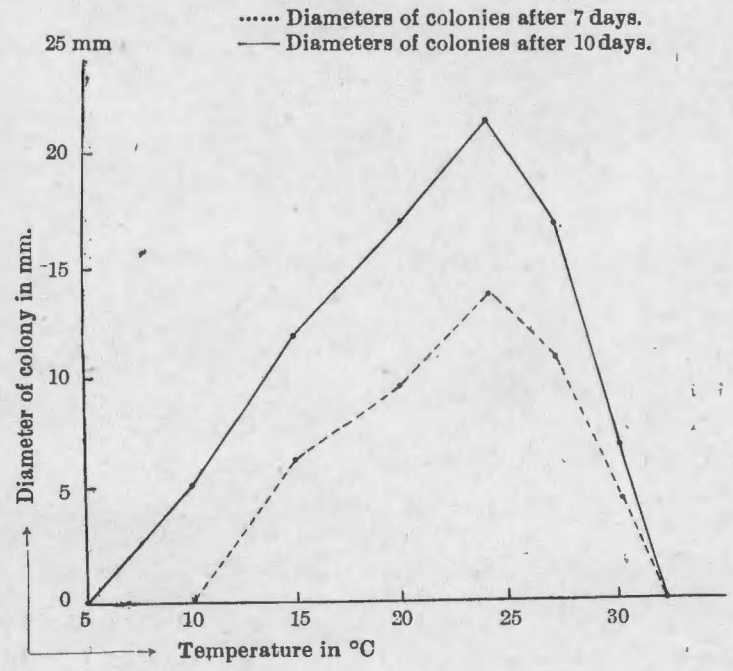
Relation of Temperature to the Mycelial Growth of *Trichloma conglobatum* (Vitt.) Sacc. in Culture.

Average of the results of 2 experiments.

Temperature	Diameter of colony in mm.				
	After 3 days	After 5 days	After 7 days	After 9 days	After 11 days
5°C	−	−	−	−	−
10°	−	−	+	+	5.0
15°	−	2.0	6.3	9.1	12.1
20°	+	5.4	9.8	13.2	17.1
24°	2.4	8.2	13.4	17.3	21.7
27°	2.5	7.2	10.9	12.9	16.3
30°	+	+	4.2	6.2	7.0
32°	−	−	+	+	+
35°	−	−	−	−	−

Figure III.

Relation of Temperature to the Mycelial Growth of *Tricholoma conglobatum* (Vitt.) Sacc. in Culture.



1. Relation of Temperature to the Growth of the Fungus Mycelium.

Each of the Petri dishes containing 15 cc. of 3% malt extract agar medium was inoculated with a piece of mycelium at the center and was incubated at different temperatures. Records were taken on the increase in diameter of the colonies. The results were as shown in Table IV; Figure III is the curve constructed from the data.

According to the results, the fungus grew between the temperatures of 10° and 30 °C. The optimum temperature for growth was in the neighborhood of 24°C. The colonies' mycelia were dense except at 15 and 30°C., which were less dense, and each colony had a very distinct margin.

2. Relation of Hydrogen-ion Concentration to the Growth of the Fungous Mycelium.

A culture medium with different hydrogen-ion concentrations was prepared by adding quantities of either N/2 solutions of hydrochloric acid or sodium hydroxide to 3% malt extract agar medium. The media of different hydrogen-ion concentrations were poured in Petri dishes and at whose conter a small mycelial fragment was inoculated. They were incubated at 24 °C and a record on the growth in diameter of the colonies was taken. Results are given in Table V with the curve in Figure IV.

The experiment showed that the fungus is able to survive between the pH of 3.8 and 10.0; the best growth occurred at pH 5.8.

Table V.

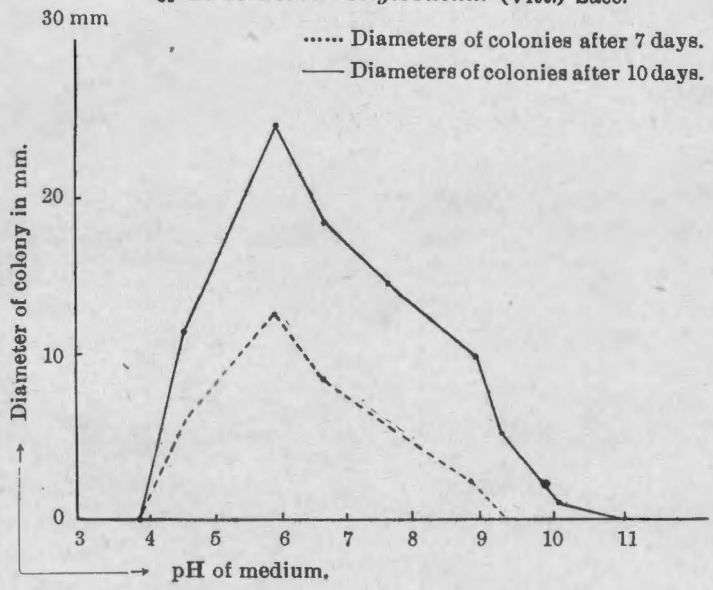
Relation of Hydrogen-ion Concentration to the Growth of Mycelium of *Tricholoma conglobatum* (Vitt.) Sacc.

Average of 5 repeated experiments.

pH of medium	Growth of mycelium in mm.				
	After 3 days	After 5 days	After 7 days	After 9 days	After 11 days
10.8	—	—	—	—	—
10.0	—	—	—	+	1.0
9.3	—	—	+	1.0	5.0
8.8	—	+	2.0	4.7	9.6
7.5	+	2.8	5.2	8.3	13.8
6.6	2.0	6.1	8.4	12.8	18.4
5.8	2.2	8.1	12.6	18.8	24.6
4.4	+	3.1	5.6	8.4	10.9
3.8	—	—	—	+	+
2.8	—	—	—	—	—

Figure IV.

Relation of Hydrogen-ion Concentration to the Growth of Mycelium of *Tricholoma conglobatum* (Vitt.) Sacc.



3. Relation of Sugar Content of Culture Medium to the Development of the Fungous Mycelium.

To find out the influence of various culture media on the growth of the fungous mycelium, eleven different kinds of agar media were prepared. Fungous colonies were allowed to develop on each of these culture media poured in Petri

Table VI.

Comparative Growth on Various Culture Media of Mycelium of *Tricholoma conglobatum* (Vitt.) Sacc.

Results after 30 days of culture at 24 °C.

Agar medium	Diameter of colony	Aerial mycelium	Density of the mycelium
Knop's solution	55 mm	—	Very sparse
„ „ + glucose	80	—	„
Sachs' solution	15	—	„
„ „ + glucose	70	—	Sparse
Potato decoction	50	±	Very dense
Soil extract + glucose	80	—	Sparse
Pfeffer's solution	20	—	Somewhat dense
Hopkin's solution	80	—	Sparse
3 % Malt extract	48	+	Dense
Richard's solution	20	—	Sparse
Currie's solution	25	+	Somewhat dense

dishes. The temperature for incubation was 24°C. and they were held for 30 days. Diameter of the colonies and a few other characters were noted; the results appear in Table VI.

The fungous mycelium made good growth on most of the media, but it was found to be extremely slow; even on the most suitable, glucose containing media, only 80 mm. of growth was noted in 30 days: Aerial mycelium occurred but weakly on only potato decoction agar, 3% malt extract agar, and Currie's solution agar, none being formed on other media. The most compact mycelial growth occurred on potato decoction agar, and was followed by 3% malt extract agar, Pfeffer's solution agar, and Currie's solution agar, while the other developed a very weak growth. Deep ridges were formed on the surface of the colonies that were cultured on potato extract agar medium. The color of the mycelium in all cases was white or milky white.

4. Relation of Sugar Content of Culture Medium to the Growth of the Mycelium.

To study this relationship, different quantities of sucrose crystals were added to 100 cc. of potato decoction agar medium. 15 cc. portions were poured into Petri dishes, inoculated with the mycelial fragment and incubated at 24°C. The result of measurements of colonies appears in Table VII. It showed that an addition of 5 g. of sucrose to 100 cc. of potato decoction was found to be most suitable for growth.

Table VII.

Relation of Sugar Content in the Culture Medium to the Mycelial Growth of *Tricholoma conglobatum* (Vitt.) Sacc.

Sugar added to 100 cc. of potato decoction agar medium	Diameter of colony	
	Sucrose	Glucose
1 g	7.0 mm	28.5 mm
5	24.5	32.3
10	10.5	28·5
15	13.0	14.5
20	10.8	8.5

5. Growth on Sawdust Culture Medium.

The sawdust culture medium was prepared the same way as it was in the case for culturing mycelium of Siitake mushroom, *Cortinellus berkeleyanus*. It consisted of 3 parts sawdust and 1 part rice bran thoroughly mixed and moistened with water and packed into large mouths glass bottles. The mouths of the bottles were plugged with cotton and were sterilized in an autoclave. An agar growth of the fungus was inoculated into the prepared sawdust medium, and it was incubated at 24°C. The rate of growth of the fungous mycelium was not rapid, but after 2–3 months the entire content of the bottle was permeated by the fungous myce-

lium. It would suggest that by this method the fungus can be made to multiply in a large volume suitable for introduction into soil.

V. Consideration on the Results.

It is safe to regard that *Tricholoma conglobatum* can grow in forests that are known to support Matutake mushroom, *Armellaria Matsutake*, with the advantage that the former can grow on much younger forest than the latter. It is of common belief that *Tricholoma conglobatum* appears within 2 or 3 years on the soil that had been burnt over with rice straw. Studies are at present being conducted at various places including the Osaka Forestry Bureau to clarify this phenomenon. Although the occurrence of the mushroom may be influenced by several interacting factors, the effect produced by burning cannot be entirely neglected. It is presumed that by burning most of the spores and mycelia of various fungi distributed near the surface of the soil are either killed or weakened by the heat. Should the spores of *Tricholoma conglobatum* be able to withstand some heating the possible explanation for the beneficial effect of burning may be forthcoming. Our results on this investigation, however, proved inadequate for giving a satisfactory explanation to this effect.

Concerning the reaction of soil, the forests of Japanese red pine usually have a pH range of about 4 to 5, which is quite strongly acid. By burning the rice straw over the soil, the ash will tend to neutralize the acid in the soil to a certain degree, although it is far from being sufficient to shift the reaction all the way to alkaline side. As the results of the experiment showed that the spores were able to germinate even in the alkaline medium, the change of reaction in the soil may have a favorable effect on the support of the fungous mycelium. A further intensive investigation, however, is required on this subject.

VI. Summary.

1. As the first in the series of studies on the principles of artificial propagation of *Tricholoma conglobatum* (VITT.) SACC., results on the germination of spores and growth of the mycelium, together with a reference on the morphology of the mushroom were presented.

2. For the growth of the mycelium, temperatures of 10 – 15 °C. were found to be minimum ; 27°C., optimum ; and about 32°C., maximum.

3. The spores were able to germinate at the pH between 3.2 and 9.0 ; a good germination at the pH 4 and 7.

4. The fungous mycelium had a minimum temperature for growth of 10 °, an optimum of 24 °, and a maximum of about 32 °C.

5. The fungous mycelium was able to develop between the pH of 3.6 and 10.0 ; the optimum was in the neighborhood of between pH 5.8 and 6.6.

6. The fungous mycelium made a good development in potato decoction agar medium. The most suitable sugar content for culture medium was about 5 %.

PLATE I.

Fig. 1. A growth of *Tricholoma conglobatum* (VITT.) SACC. in a natural habitat. Photographed November, 1934, at the test-plot of Mr. H. Seno, Misao-Yama, Okayama-City.

PLATE II.

Fig. 2. Sporophores of *Tricholoma conglobatum*. 8/10.

Fig. 3. A group of sporophores of *Tricholoma conglobatum*. Photographed November, 1933, at the experimental plot of Okayama Forestry Station, Misao-Yama Okayama-City.

PLATE III.

Fig. 4. A cluster of *Tricholoma conglobatum.* ×6/10.

Fig. 5. Longitudinal and cross sections of the sporophores of *Tricholoma conglobatum.* ×6/10.

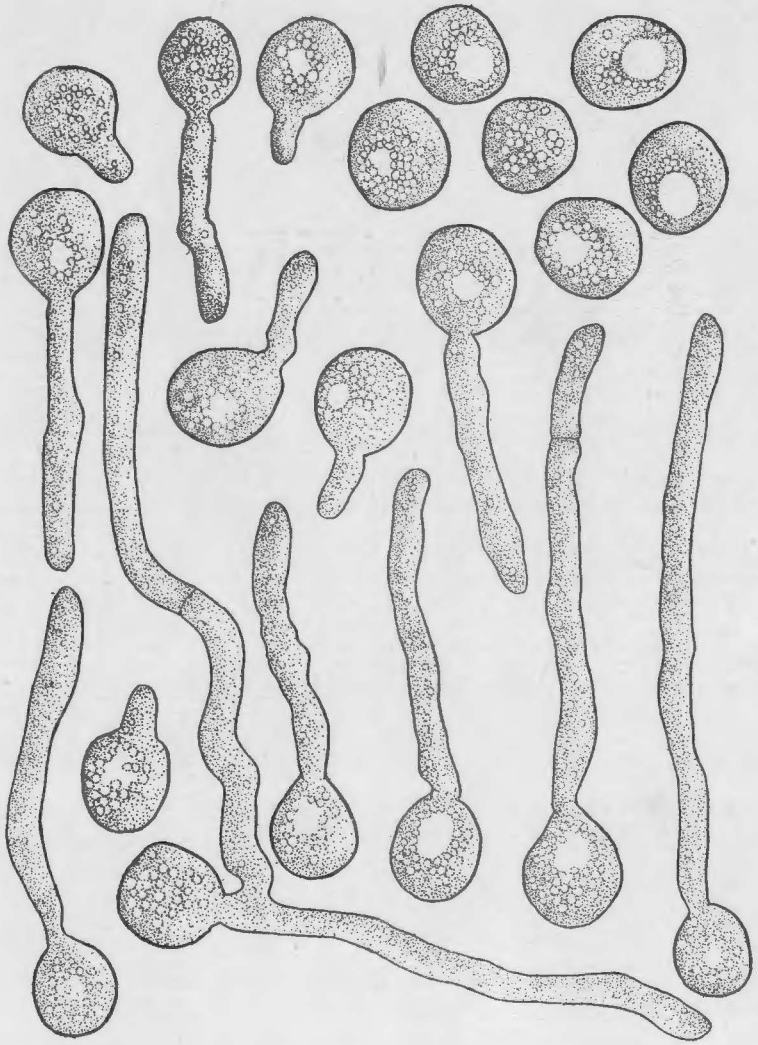


Fig. 6. The germinated spores of *Tricholoma conglobatum* on 3 % malt extract agar medium; after 3 days' incubation at 27°C. ×1800.

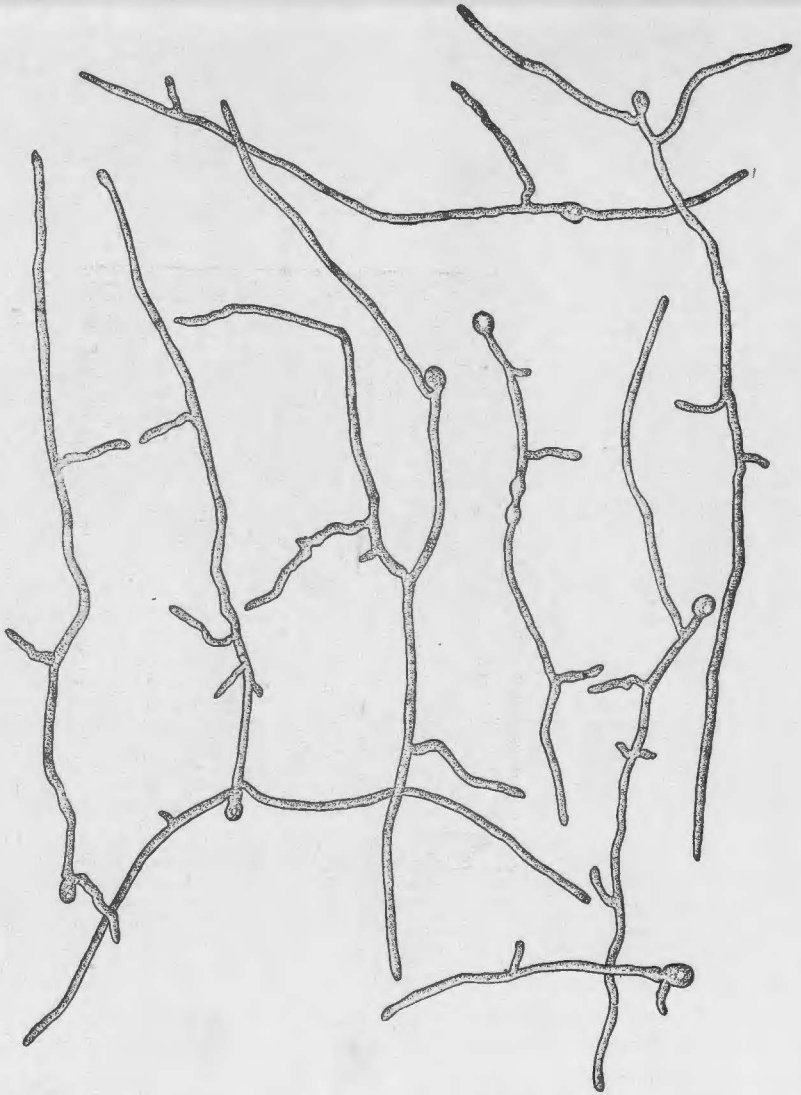


Fig. 7. The germinated spores of *Tricholoma conglobatum* on 3 % malt extract agar medium; after 7 days' incubation at 27 °C. ×400.